Marginal Zone SIGN-R1(+) Macrophages are Essential for the Maturation of Germinal Centre B cells in the Spleen

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Abstract

The germinal centre (GC) response is an essential component of adaptive immunity, crucial for the generation of high-affinity antibodies, long-lived memory and plasma cells and is the basis for most current vaccines. While the dynamics of cognate B and T lymphocytes during the GC response are extensively studied, the roles of resident tissue macrophages in this process are less well understood, particularly in the spleen. A unique compartment to the spleen, called the marginal (MZ), is known to be important for adaptive immunity and harbours at least two distinct MZ-resident macrophage subsets. Both MZ macrophage populations are essential for capturing and preventing systemic dissemination of pathogens. However, their individual functions and potential role in adaptive immunity are not established.

Here we used pharmacologic and genetic approaches to deplete SIGN-R1+ MZ macrophages and showed that these cells were specifically required for the optimal development of germinal centre responses in the spleen. SIGN-R1+ macrophages were dispensable for the initial GC establishment but were required for optimal maintenance of the response. The GC defect could be partially corrected by boosting T follicular helper (Tfh) cell responses or by inducing Tfh help prior to macrophage ablation. Furthermore, in the absence of SIGN-R1+ macrophages, 33D1+ DCs, a key population involved in Tfh priming, was displaced from the interfollicular regions to the MZ. Reconstituting the SIGN-R1+ subset restored DC positioning and rescued the GC phenotype. This work advances our understanding of how the splenic marginal zone regulates adaptive immunity, highlights the functional diversification of MZ macrophages, and provides the first genetic mouse model allowing for the specific depletion and modification of SIGN-R1+ cells.

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1. Introduction

1.1. Functions of the spleen

The spleen is the largest secondary lymphoid organ in the body. Situated in the abdominal cavity underneath the diaphragm, it is the main site where senescent or damaged red blood cells are filtered and iron is recycled, a reservoir for erythrocytes and monocyte progenitors and, importantly, it is a site for filtering systemic antigens and mounting an adaptive immune response against pathogens that have breached the mucosal barriers and have entered the circulation (Bronte & Pittet 2013, Kim 2010, Mebius & Kraal 2005). Surgical removal of the spleen after splenic injury or to ameliorate conditions such as anaemia, sickle cell disease and lymphoproliferative disorders, is associated with an increased risk of sepsis, as well as reduced recovery from bacterial and viral infections such as pneumonia and meningitis (Crary & Buchanan 2009, Deodhar et al 1993, Kyaw et al 2006). Furthermore, the spleen may represent a promising vaccine target as highlighted by a recent study showing that intravenous administration of BCG vaccine can induce a more potent immune response in comparison to intradermal or aerosol administration and provide almost absolute protection against tuberculosis infection in macaques (Darrah et al 2020). Therefore, understanding the immune processes in the spleen and the mechanisms that have evolved specifically against blood-borne pathogens could potentially help us design better therapies and vaccines in the future.

1.2. Overall architecture of the spleen compared to lymph nodes

While the spleen shares many similarities with lymph nodes (LNs) in terms of immune cell composition, organisation and function, there are also major structural differences

between the two lymphoid organs. Similar to LNs, the spleen contains a lymphoid tissue (Fig. 1), the white pulp, which is organized into B cell follicles and T cell zones and which is the main site where adaptive immunity is induced. However, in addition to this compartment, the spleen includes 2 additional distinct regions; the red pulp - a meshwork of fibroblasts and reticular fibres where blood flows freely and is filtered from old and damaged red blood cells before returning to the circulation and the marginal zone (MZ), a narrow compartment that lays between the red and the white pulp which harbours specialised immunes cells integral to its immune functions (Mebius & Kraal 2005).

Lymphocyte entry into the LNs is a well-described process that requires integrins for adhesion, rolling and transmigration through the high endothelial venules (HEVs) (Sixt et al 2006, Takeda et al 2017). In contrast, the exact entry sites or even the role of integrins in lymphocyte migration into the white pulp are less clear (Lo et al 2003, Manevich-Mendelson et al 2010, Nolte et al 2002). A recent study using 2-photon live imaging revealed that T cell migration towards the T zone of the spleen is facilitated by a one-directional migration along the radial arterial vessels that act as tracks onto which T cells latch. This movement allows the cells to enter T zones via the bridging channels in a CCR7-dependent manner, a process that did not require but was accelerated by the integrins LFA-1 and VLA-4 (Chauveau et al 2020). While it remains to be directly established whether B cells also use these tracks for entry, B cells have been observed to migrate from the bridging channels towards the B cell follicles (Bajenoff et al 2008), suggesting this might be the case. The MZ zone is another potential site for B cell entry as B cells were found in the inner MZ as early as 15 min after transfer, and this was dependent on the integrins LFA-1 and α4β1 as well as on GPCRs (Lo et al 2003).

Furthermore, follicular B cells have also been observed to exit the follicles via the MZ (Arnon et al 2013) further suggesting the MZ-follicle axis as a route that facilitates B cell recirculation.

Once inside the white pulp, the migration within the B cell and T cell compartments is conserved between the spleen and LNs (Fig.1, Fig.2). Newly arrived B and T lymphocytes actively migrate towards their corresponding zones, guided by the stromal cell-derived chemokine gradients of CXCL13 in the B cell follicle and CCL19 and CCL21 in the T cell zone which bind CXCR5 and CCR7 on B and T cells respectively (Mebius & Kraal 2005). Exit of the cells from the white pulp, similarly to LNs, is dependent on the surface expression of the G-protein-coupled receptor S1PR1, which responds to its ligand sphingosine-1-phosphate (S1P) (Cyster & Schwab 2012, Mandala et al 2002). S1P concentrations are high in the blood and lymph but maintained low in the white pulp and interstitial fluids of the spleen and LNs, thus promoting S1PR1+ cell egress (Cyster & Schwab 2012, Mandala et al 2002). Crucial for the recirculation of lymphocytes is the high sensitivity of S1PR1 to ligand-mediated downregulation due to which blood-borne lymphocytes quickly lose S1PR1 from their surface and regain the ability to re-enter lymphoid tissues (Arnon et al 2011).

A major structural difference between LNs and spleen is that, while in the lymph node antigen and lymphocytes arrive separately, via the afferent lymphatics and HEVs respectively, in the spleen cells and antigen arrive together from the blood under the high pressure of the circulation. Since many of the blood antigens will pass directly through the MZ region (Mebius & Kraal 2005), the MZ represents an important site for immune monitoring.

1.3. Marginal zone of the spleen

The marginal zone (MZ) of the spleens is an important site for trapping and filtering blood-borne pathogens and promoting an immune response against them (Mebius & Kraal 2005) as well as clearing apoptotic cells and preventing autoimmune responses (Prabagar et al 2013, Wermeling et al 2007). It consists of a thin layer of specialized MZ-resident cells at the interface between the red and the white pulp (Mebius & Kraal 2005). Upon entry into the spleen part of the blood is initially slowed down within the thin vessels of the marginal sinus, at the base of the MZ, before eventually spilling into the MZ and towards the red pulp. This provides an important opportunity for MZ-resident cells to screen the blood for pathogens (Mebius & Kraal 2005, Schmidt et al 1993). Importantly, the marginal sinus also serves as a physical barrier that prevents free access of cells, fluids and large molecules into the white pulp, while molecules smaller than 70 kDa can enter via conduits that permeate both the B cell follicles and the T cell zone (Mebius & Kraal 2005, Nolte et al 2003).

1.4. Marginal zone-resident cells

The MZ contains several lymphoid and non-lymphoid resident cells. The main known subsets include MZ-resident B cells (MZBs), marginal zone macrophages and a population of CD8+ DCs (Idoyaga et al 2009, Martin & Kearney 2002, Qiu et al 2009). In addition, metallophilic macrophages, located underneath the marginal sinus within the white pulp, also can access the marginal zone compartment via dendritic processes (Gordon et al 2014, Martinez-Pomares & Gordon 2012) and share similar functions with MZ-resident macrophages in immune surveillance.

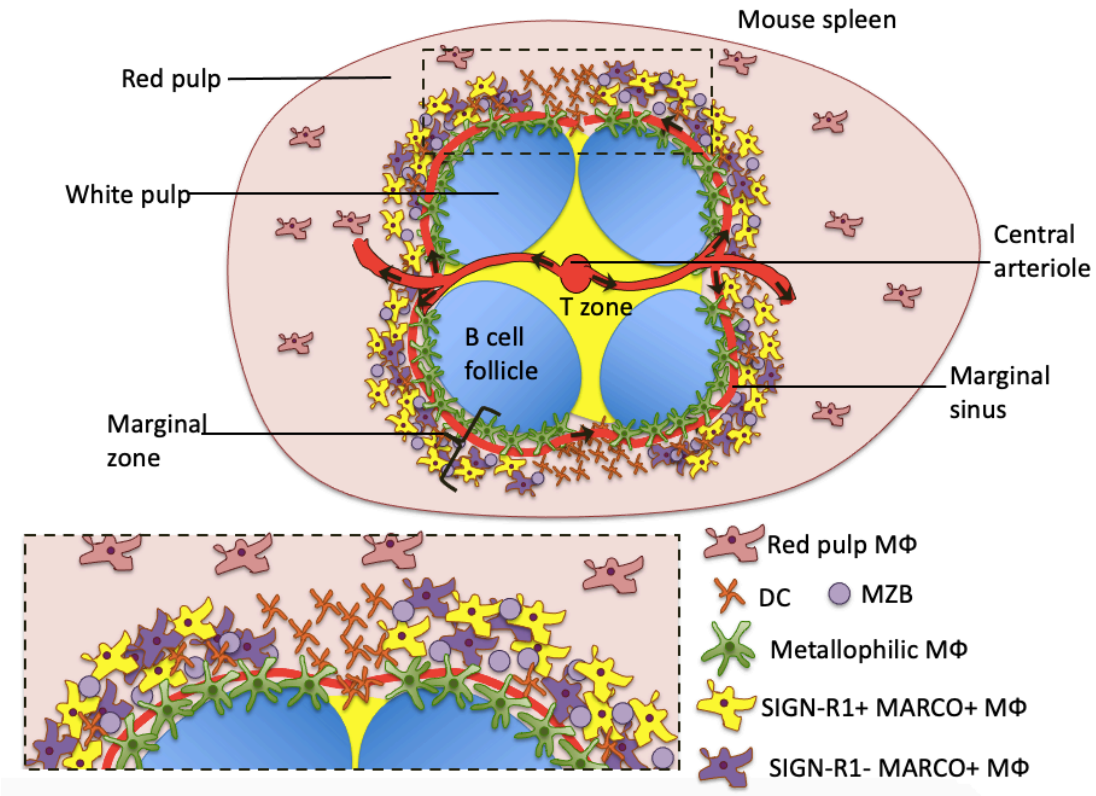


Figure 1. Structure of the mouse spleen.

The spleen is divided into red pulp, marginal zone and white pulp, further organised into B cell follicles and T cell zone. Blood flows (arrows) from the central arteriole into the marginal sinus and the red pulp, and eventually joins the open blood flow in the red pulp. Bottom panel, zoomed schematic of the MZ. There are three main macrophage (MΦ) subsets associated with the MZ region - metallophilic macrophages, which reside underneath the marginal sinus and can access the MZ via dendritic processes, and two marginal zone macrophage subtypes - SIGN-R1+MARCO+ double positive cells, and SIGNR-1- MARCO+ single positive cells.

1.4.1 Marginal zone B cells

MZBs have $IgM^{hi}IgD^{low}CD21^{hi}CD23^{-}CD1d^{hi}$ phenotype and innate-like features, as they recognise conserved molecular microbial patterns via their polyreactive BCR receptors, high levels of TLR and complement receptors (Cerutti et al 2013). Therefore, MZBs are directly involved in blood immune surveillance (Martin & Kearney 2002, Pillai et al 2005). In response to blood-borne pathogens MZBs can quickly differentiate

into short-lived plasmablasts that secrete low-affinity antibodies before the development of the slower adaptive immune response (Martin & Kearney 2002). In recent years it has become clear that MZB localisation in the MZ is not fixed. Instead, they constantly shuttle between the MZ region and the follicles (Arnon et al 2013, Cinamon et al 2008). MZBs acquire antibody-coated antigens captured from MZ macrophages and transfer them to FDCs within the white pulp (Prokopec et al 2016, You et al 2011). This migration is guided by two opposing forces – on one hand the S1P gradient from the MZ and the blood flow itself pull MZBs towards the red pulp and on the other, CXCL13 attracts them towards the B cell follicles, while the integrins αLβ2 (LFA-1) and α4β1 (VLA-4) tether them to ICAM-1 and VCAM-1 receptors expressed in the MZ (Cinamon et al 2004, Lu & Cyster 2002).

1.4.2 Marginal zone macrophages

There are three macrophage subsets associated with the MZ region, distinguished by their surface marker expression and localisation – metallophilic macrophages and two subsets of marginal zone macrophages. Metallophilic macrophages are found underneath the marginal sinus at the periphery of the B cell follicles and their ability to extend dendritic processes across the marginal sinus barrier and into the MZ allows them to act as a bridge between the two compartments (Gordon et al 2014, Martinez-Pomares & Gordon 2012) (Fig.1). In contrast, the MZ macrophages, as the name suggests, are situated within the MZ itself and express the macrophage receptor with collagenous structure (MARCO). MARCO+ macrophages can be further subdivided into two populations depending on their expression of the lectin receptor SIGN-R1 – there are double positive SIGN-R1+ MARCO+ macrophages and single positive SIGN-R1- MARCO+ cells (Gordon et al 2014, You et al 2011)(Fig.1). In this thesis SIGN-R1+ MARCO+ macrophages are referred to as “SIGN-R1” cells and SIGN-R1-

MARCO+ macrophages are referred to as “MARCO” cells. While it is possible that SIGN-R1 cells are derived from MARCO or represent a different activation state of these cells, previous research suggests that they are two distinct populations, as they have different developmental requirements and kinetics. SIGN-R1 but not MARCO MZ macrophages require M-CSF (Ito et al 1999, Takahashi et al 1994) and depend on the presence of MZB cells for maintenance (Takahashi et al, 1994; Ito et al, 1999). Furthermore, MARCO cells also develop much earlier than SIGN-R1 (Chen et al 2005). In mice, interspersed MARCO+ cells are present in the spleen at birth and by day 3 are already organised into the typical circular pattern surrounding the marginal sinus (Chen et al 2005). Only at day 7 SIGN-R1 cells start to appear, maturing to the adult pattern of distribution at around day 20 after birth (Chen et al 2005). Similarly, MARCO macrophages are also the first to repopulate the MZ after total macrophage depletion with clodronate liposomes (CLL) in adult mice (van Rooijen et al 1989).

All three macrophage subsets are important for immune surveillance against blood-borne bacteria and viruses, activation of the innate immune response and the induction of peripheral tolerance (Gordon et al 2014, Martinez-Pomares & Gordon 2012). They bind a wide range of bacterial, viral and apoptotic antigens (Kang et al 2004, Koppel et al 2005, Lanoue et al 2004, McGaha & Karlsson 2016, Nagaoka et al 2005) and have been suggested to be the major producers of type I interferon in response to viral infection in the spleen (Eloranta & Alm 1999, Schwandt et al 2012). In agreement with the latter, total macrophage ablation leads to reduced expression of proinflammatory cytokines such as IFNα, IL-27 and IL-6 (Veninga et al 2015). Depletion of all 3 subsets also correlates with loss of pathogen trapping in the MZ, resulting in overwhelming systemic spread of pathogens as shown for example after infection with lymphocytic

choriomeningitis virus (LCMV) (Seiler et al 1997) or *Listeria monocytogenes* (Aichele et al 2003)(Perez et al 2017). Similarly, MZ macrophages are essential for the clearance and induction of tolerance to apoptotic cells (Miyake et al 2007, Ravishankar et al 2014), with depletion of all three subsets leading to a delayed clearance, altered pattern of antigen deposition on DCs and onset of autoimmunity to apoptotic cell antigens (Miyake et al 2007). Additionally, macrophage depletion correlates with disorganisation of other immune cell subsets such as NK cells, CD8+ and CD11b+ leukocytes (Perez et al 2017) and leads to abolished CTL response to adenovirus (Backer et al 2010).

Our knowledge of the individual functions of the different MZ macrophage populations however is limited, partly due to lack of models that can reliably remove only one of the subsets. For example, some studies have used CD169-DTR mice in an effort to identify the specific roles of metallophilic macrophages (Perez et al 2017). Unfortunately, DT treatment in this model also leads to the depletion of both SIGN-R1 and MARCO macrophage subsets (Miyake et al 2007, Ravishankar et al 2012) and therefore the effects observed cannot be reliably attributed to either subset.

1.4.3 Metallophilic macrophages

Metallophilic macrophages, named after their affinity for silver staining (Dullmann et al 2000), express the sialic acid binding Ig-like lectin (Siglec) receptor Siglec-1, also known as CD169 or sialoadhesin (Kraal & Janse 1986, Martinez-Pomares & Gordon 2012, Oetke et al 2006). CD169+ macrophages are efficient at internalising pathogens and account for at least half of Listeria monocytogenes captured in the spleen after infection (Perez et al 2017). Furthermore, CD169+ macrophages have been proposed to support localised viral replication (Honke et al 2011). This is because they are less

responsive to type I interferon-induced suppression of viral replication resulting in viral particle accumulation specifically in this subset, while viral growth within other macrophages, including those in the marginal zone, liver, kidney and brain, is suppressed. One hypothesis is that this may provide a necessary and controlled supply of antigen that is important for supporting the adaptive immune response in the spleen (Honke et al 2011). Similarly, CD169+ macrophages were shown to be specifically susceptible to infection with *S. pneumoniae* that evaded clearance by other macrophages and proliferated within CD169+ cells in the spleen (Ercoli et al 2018). However, in this case the surviving bacteria could escape back into the circulation leading to late onset of sepsis (Ercoli et al 2018), highlighting the context-dependent function of CD169+ macrophages in infection.

Similarly, in LNs a population of CD169+ macrophages at the subcapsular sinus (SCS macrophages) also specifically facilitates VSV replication (Iannacone et al 2010). In contrast to the spleen however, in LNs removal of SCS macrophages does not inhibit the adaptive immune response to VSV, but instead leads to a reduced type I interferon production and the subsequent spread of infection to the peripheral nervous system (Iannacone et al 2010). This highlights that while resident macrophage populations in LNs and spleen can have phenotypic and functional similarities, their functions are not directly analogous.

Several studies also suggest metallophilic macrophages might be a promising vaccine target. Injecting ovalbumin-conjugated antibody against CD169 (CD169-OVA) leads to a potent humoral response (Veninga et al 2015), as well as a significant antigen deposition on CD8+ DCs, cross-presentation and activation of CD8+ T cells (Backer et

al 2010). Furthermore, glycan ligand-decorated liposomes, used to target CD169+ cells, can induce invariant natural killer T (iNKT) cell priming (Chen et al 2012, Kawasaki et al 2013). Targeting antigen to CD169 in the spleen can also induce secretion of pro-inflammatory cytokines as intravenous injection of sialylated bacteria into WT or CD169 KO mice lead to IL-6, IFN-β and TNF-α production that was dependent both on the bacterial sialylation as well as CD169 expression (Klaas et al 2012). Similarly, in LNs SCS macrophages have been established to cross-present dead tumour cells directly to CD8+ T cells (Asano et al 2011), present lipids to activate iNKT cells (Barral et al 2010), and capture opsonized antigens and viral particles from the lymph and translocate them to B cells (Junt et al 2007, Phan et al 2007)(Carrasco & Batista 2007). Taken together, these data suggest that splenic CD169 macrophages have the potential to be potent inducers of both innate and adaptive immunity.

1.4.4 SIGN-R1 macrophages

The marginal zone macrophage subset expressing dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) homolog, SIGN-related 1 (SIGN-R1) receptor is well known for its capacity to efficiently bind blood-borne antigens. SIGN-R1 is a C-type lectin, a type of pattern-recognition molecule, and one of five murine homologues of the human DC-SIGN gene found on human dendritic cells (Figdor et al 2002, Park et al 2001). Apart from MZ macrophages, SIGN-R1 is also expressed on murine peritoneal and LN medulla-resident macrophages as well as on liver sinusoidal endothelial cells (Geijtenbeek et al 2002, Gonzalez et al 2010, Taylor et al 2004). A recent study also suggested SIGN-R1 could be expressed by a subset of inflammatory DCs during influenza virus infection (Palomino-Segura et al 2019). Some studies propose that SIGN-R1 receptor may bind the C1q complement subunit and facilitate C3 convertase activation without the requirement for Ig or immune complexes (Kang

et al 2006, Prabagar et al 2013). This possibility was supported by structural analysis, showing 2 independent binding sites that can attach C1q and a sugar moiety simultaneously (Silva-Martin et al 2014). Thus, the SIGN-R1 receptor can potentially capture and then mark pathogens for recognition by immune cells expressing complement receptors, such as MZBs, DCs and macrophages.

SIGN-R1 can recognise a multitude of antigens. *In vitro* SIGN-R1-transfected cells bind fungal, bacterial and viral pathogens such as yeast (Taylor et al 2004), *S. aureus* (Elomaa et al 1995), HIV-1 (Geijtenbeek et al 2002) as well as adhesion molecules such as ICAM-2 (Geijtenbeek et al 2002), suggesting these interactions could occur in vivo. Specifically on MZ macrophages, SIGN-R1 is important for capturing blood-borne bacteria such as *S. pneumoniae* (Kang et al 2004, Koppel et al 2005, Lanoue et al 2004), *E.coli*, *Salmonella* (Nagaoka et al 2005), as well as for clearing apoptotic cells and promoting immune tolerance (Prabagar et al 2013). SIGN-R1 is essential for binding and capturing bacterial capsular polysaccharides (CPS) as pre-treatment with the anti-SIGN-R1 antibody, 22D1, which transiently depletes the SIGN-R1 receptor from the cell surface leaving the cells intact, abolishes CPS accumulation in the MZ (Kang et al 2004).

A role for SIGN-R1 in adaptive immunity in the spleen has not yet been shown - SIGN-R1 knockout mice have normal IgG response to the model antigen TNP-KLH (Koppel et al 2005) and targeting OVA antigen using SIGN-R1-specific antibodies does not promote CD8+ T cell responses (Backer et al 2010). However, while these studies do not uncover a defined function for the SIGN-R1 molecule itself, our understanding of the specific functions of SIGN-R1 expressing MZ macrophages is limited.

1.4.5 MARCO macrophages

Macrophage receptor with collagenous structure (MARCO) is expressed in the splenic MZ and the medullary region of LNs and can bind and induce phagocytosis of Gram-negative and Gram-positive bacteria such as *E.coli* and *S.aureus*, independently of SIGN-R1 and CD169 receptors (Elomaa et al 1995, Elshourbagy et al 2000, Ito et al 1999). Mice lacking MARCO receptor have an increased bacterial burden and reduced survival in a bacterial pneumonia model (Arredouani et al 2004). Similar to SIGN-R1, the MARCO receptor has a role in directly capturing apoptotic cells, preventing autoimmunity (Wermeling et al 2007). Highlighting the importance of these receptors in immune tolerance, in BXD2 mice, which develop spontaneous lupus-like autoimmune disease, there is a significant reduction in both MARCO+ and SIGN-R1+ splenic macrophages, associated with increased apoptotic cell translocation to the follicles and accumulation of self-antibodies (Li et al 2013). Targeting antigen to MARCO MZ macrophages via OVA-conjugated anti-MARCO antibody, similarly to CD169 targeting, leads to efficient priming of CD8 T cells (Backer et al 2010), suggesting MARCO cells could be a useful vaccine target.

1.4.6 Interactions between MZ-resident cells

MZ-resident cells are tightly confined within the MZ region suggesting these subsets are constantly interacting with each other. Indeed, multiple studies suggest that there is a complex dependency between the different populations. Direct contact between MARCO macrophages and MZB cells has been suggested to play a role in the maintenance of the MZB subset as specific deletion of the negative signalling inositol phosphatase, SH2-containing inositol-5-phosphatase 1 (SHIP) in myeloid cells, which causes the reorganisation of MARCO macrophages from the MZ compartment to the

red pulp, also leads to the significant reduction and displacement of MZB cells, while metallophilic macrophages are still present (Karlsson et al 2003). Furthermore, blocking MARCO receptor itself induces MZB cell migration from the MZ into the follicles (Karlsson et al 2003), suggesting constant interaction supports MZB cell location within the MZ region. The MZB-MARCO receptor interaction however is not essential, as mice lacking the MARCO receptor alone still develop MZB, SIGN-R1 and CD169 cell subsets in the correct compartments, although with slightly delayed kinetics (Chen et al 2005). It is thus possible that loss of MARCO receptor-independent signals or acute activation in response to anti-MARCO antibodies in the above studies are responsible for the loss of MZB cells in the MZ.

Importantly, SIGN-R1 macrophages depend on constant interaction with MZB cells. For example, CD19 KO mice, in which B cells are severely impaired in their ability to differentiate into MZB cells, (Martin & Kearney 2000, You et al 2009), lack SIGN-R1 but not MARCO or CD169 macrophages (You et al 2009). This interaction is also important for SIGN-R1 maintenance as transient loss of MZB cells due to downregulation of S1PR1 with the drug FTY720, can lead to transient loss of SIGN-R1 cells (You et al 2011), while gradual loss of MZB cells in old mice (Birjandi et al 2011) or in CD70TG mice in which B cells are gradually reduced (Nolte et al 2004), correlates with reduced number of SIGN-R1 cells (Birjandi et al 2011). In contrast, the presence of MZB and MARCO cells within the marginal zone is independent of SIGN-R1 or CD169 macrophages as in M-SCF-deficient (op/op) mice, which lack CD169 and SIGN-R1 macrophages, there are still MZB and MARCO cells (Ito et al 1999, Karlsson et al 2003). Thus, maintenance of the MZ architecture and function requires specific interactions between the MZ-resident subsets.

1.5. The germinal centre response in the spleen

Germinal centres (GC) are transient structures that form in lymphoid follicles where antigen-activated B cells proliferate extensively, undergo somatic hypermutation and selection, which results in the generation of high affinity antibodies, long-lived plasma cells and memory cells that protect against secondary encounter with the antigen (Victora & Nussenzweig 2012). GC regulation is a complex process that requires coordination of multiple cell types that migrate between specific GC microenvironments in a time-sensitive manner (Cyster & Allen 2019, Stebegg et al 2018). Dysregulation of this process can result in autoimmunity, cancer and immunodeficiency (Bousfiha et al 2020, DeFranco 2016, Elsner & Shlomchik 2020).

1.5.1 B cell activation and migration

Upon entry into the follicular compartment, naïve B cells begin to migrate in a random manner, scanning for antigen on FDCs (Suzuki et al 2009). Cognate antigen binding triggers a series of migratory events that guides B cells into specific microenvironments (Fig.1) (Cyster 2010, Gatto & Brink 2013). Shortly after antigen binding B cells upregulate the surface molecule CD69, which binds to and inhibits S1PR1, a master regulator of lymphocyte egress from lymphoid tissues that is necessary for their egress into the circulation. The interaction between CD69 and S1PR1 facilitates initial S1PR1 internalisation and degradation, thus ensuring activated cells are retained within the lymphoid tissue, where their subsequent maturation can potentially occur (Bankovich et al 2010, Shiow et al 2006). At the same time, the chemotactic receptor EBI2 is upregulated, driving activated B cell migration towards the perimeter of the follicle and the interfollicular regions, where a gradient of the EBI2 ligand, 7α,25-OHC is established by stromal cells (Lu et al 2017, Yi et al 2012). The purpose of this initial

migration, which occurs as early as 1 hour after activation (Gatto et al 2009, Kelly et al 2011, Pereira et al 2009), is not fully understood, but could potentially facilitate the acquisition of new antigen from the metallophilic macrophages or dendritic cells that reside within the bridging channels (Gatto & Brink 2013). Around 6 hours after antigen-mediated activation B cells upregulate the chemotactic receptor CCR7, which enhances migration towards the T zone, where its ligands CCL19 and CCL21 are abundant (Kelly et al 2011). In addition, FDC-derived CXCL13 continuously activates the chemotactic receptor CXCR5 and enhances movement towards the B cell follicles. As a result of these competing gradients, EBI2+ CXCR5+ CCR7+ B cells adopt an intermediate position residing at the border between the T and B cell zones (Kelly et al 2011, Okada et al 2005) (Fig.2). Here activated B cells with the highest relative affinity for the antigen come in contact with and present more peptide-MHCII to cognate T cells than lower affinity B cells, forming stable B-T interactions, which in turn promotes further B cell activation (Schwickert et al 2011). Within 2-3 days, CCR7 is downregulated, such that EBI2 becomes dominant again, leading B cells back to the interfollicular and outer follicular regions, where they proliferate extensively (Coffey et al 2009). These cells upregulate surface markers such as GL7 and CCR6 (Taylor et al 2012) and are thought to serve as the precursors of GC B cells, therefore referred to as pre-GCs (Kerfoot et al 2011, Kitano et al 2011). A fraction of these cells upregulates the master regulator of GC differentiation, BCL-6, and thus commit to the GC B cell differentiation pathway (Kerfoot et al 2011, Kitano et al 2011, Taylor et al 2012). BCL-6 directly downregulates EBI2 (Shaffer et al 2001) which reduces the attraction of activated B cells to the follicular perimeter and interfollicular regions, while CXCR5 expression drives them to preferentially cluster near CXCL13-producing FDCs in the centre of the follicles (Gatto et al 2009, Pereira et al 2009). This confinement is further

supported by S1PR2 upregulation, which acts to repel cells away from its ligand S1P, which is more abundant at the perimeter of the follicles (Green & Cyster 2012, Green et al 2011). GC cells establishment in the centre of the follicles displaces naïve IgM+ IgD+ B cells (partially due to naïve B cells being negative for S1PR2), which can be visualised by histology as an IgD^{low} area in the centre of the follicle. GCs will then proliferate extensively and around day 7 further segregate into a light and a dark zone where selection and hypermutation take place, respectively (De Silva & Klein 2015). Following multiple rounds of T-dependent selection and proliferation the high clonality of early GCs will be drastically reduced in mature germinal centres, with highest affinity clones preferentially surviving the selection process to ultimately form long-term memory cells and plasma cells (Tas et al 2016).

This tightly regulated series of migratory events is essential for the sequential delivery of activation signals and subsequently for the optimal development of the adaptive immune response. For example, in mice in which EBi2 is selectively depleted in B cells, the early IgG1 plasma cell response is significantly reduced due to the cells being unable to cluster in the interfollicular and outer follicular regions and instead positioning in the centre of the follicle to directly form germinal centres (Gatto et al 2009, Pereira et al 2009). Furthermore, when activated B cells are made to overexpress EBI2, preventing downregulation of the molecule, their incorporation in the GC reaction is severely reduced and B cells preferentially differentiate into plasmablasts (Gatto et al 2009, Pereira et al 2009).

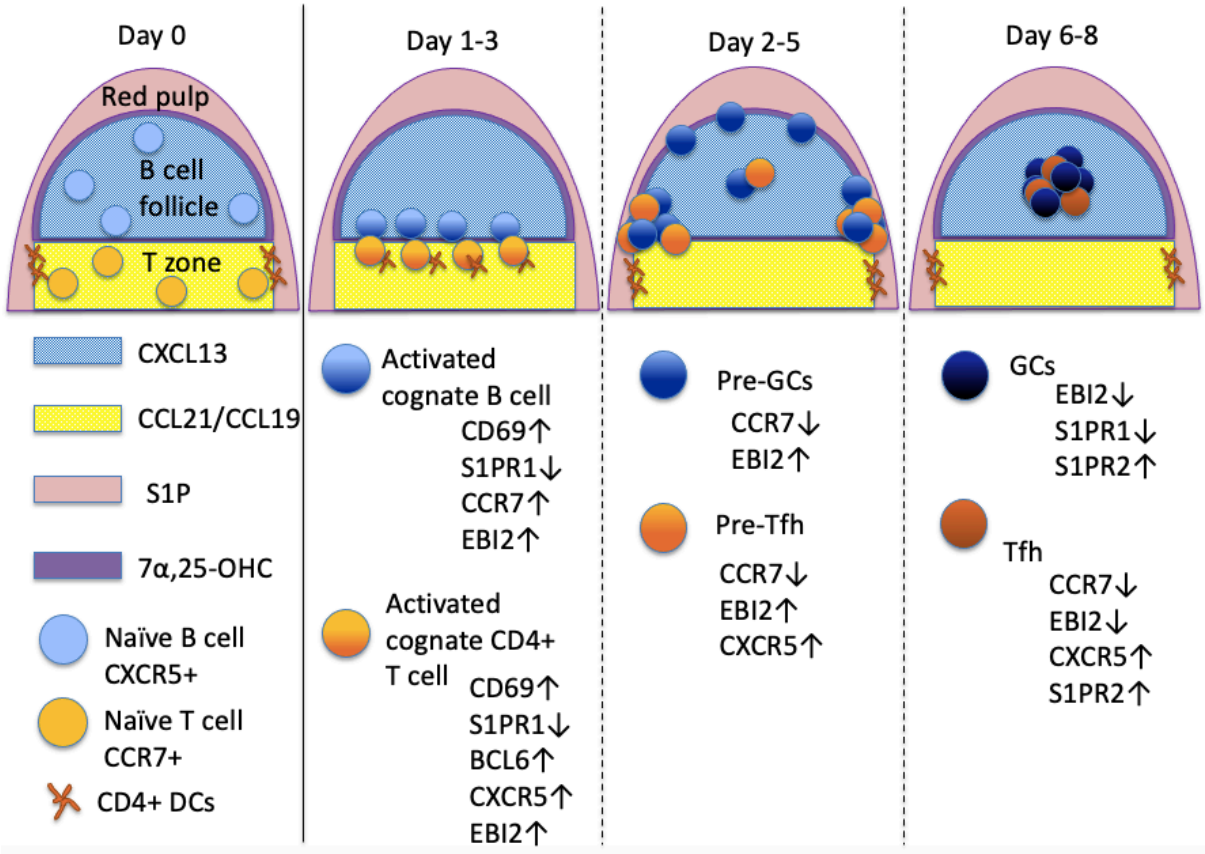


Figure 2. Migration of B cells and CD4+ T cells after activation.

Naïve circulating B and T cells migrate to their designated compartments in the splenic white pulp guided by the expression of the chemokine receptors CXCR5 and CCR7 towards CXCL13 and CCL21/CCL19 ligands present in the B cell follicles and T cell zone, respectively. After immunisation T cells are activated first by activated CD4+ DCs that migrate from the interfollicular regions to the outer T zone. Activated T cells upregulate the chemokine receptor EBI2 and CXCR5 guiding them to accumulate at the outer T zone and B/T border. A secondary T cell priming by activated cognate B cells occurs at the B/T border, leading to the formation of stable B:T conjugates. Within ~2 days post immunisation, B and T cells migrate towards the interfollicular regions where they undergo clonal expansion. B cells also migrate towards the periphery of the follicles, guided by EBI2, where they may acquire more antigen. After day 5, B and T cells downregulate EBI2 and migrate towards the centre of the follicles to establish the GC reaction. S1PR2 upregulation at this stage helps to confine them within the GC structure by inhibiting migration towards its ligand S1P at the periphery of the follicles (Cyster 2010, Gatto & Brink 2013, Moriyama et al 2014, Vinuesa et al 2016).

1.5.2 T cell activation and migration

T follicular helper (Tfh) cells are crucial for the formation and maintenance of GC responses. Similar to GC B cells, optimal differentiation of Tfh cells requires spatiotemporal control, with sequential delivery of activation signals within specific niches (Vinuesa et al 2016). There are two main activation steps important for Tfh priming – an initial priming event by activated DCs within the T zone and a secondary priming step by activated B cells at the border between the B cell follicles and T cell zone (Deenick et al 2010, Li et al 2016). After T cell priming activated T cells migrate towards the interfollicular regions where they undergo clonal expansion (Coffey et al 2009, Cyster 2010, Vinuesa et al 2016) before migrating finally towards the GC structure at the follicular centre (Vinuesa et al 2016). Once within the GC, Tfh cells depend on persistent TCR stimulation by GC B cells for survival as well as GC B cells depend on Tfh help to continue the cycle of proliferation, hypermutation and selection (Baumjohann et al 2013, Deenick et al 2010, Victora & Nussenzweig 2012).

Within the first 12 hours after immunsation T cells upregulate the chemokine receptor EBI2 (Li et al 2016). EBI2-mediated chemotaxis towards its ligand, 7α,25-OHC (Lu et al 2017), is essential for localising cognate T cells at the outer T zone where they can be primed by CD4+ DCs (Li et al 2016). EBI2 KO T cells fail to accumulate in the outer T zone which is associated with a significant reduction of Tfh numbers and a poor GC response (Li et al 2016). Within 24 to 72 hours after DC activation T cells start expressing the master regulator of Tfh differentiation, BCL6, as well as the chemokine receptor CXCR5, acquiring the typical CXCR5+ BCL6+ Tfh phenotype (Baumjohann et al 2011, Kitano et al 2011) and localise at the B/T border where the secondary priming event by cognate B cells takes place (Li et al 2016). Cognate B:T interactions

are essential for Tfh differentiation and maintenance (Deenick et al 2010). These conjugates are stable and can last between 10 to more than 60 minutes (Okada et al 2005) with their stability depending on the adaptor proteins SAP and SLAM family members such as CD84 and Ly108 (Qi 2012, Qi et al 2008, Vinuesa et al 2016). Defects in cognate B:T interactions, for example in the absence of SAP, lead to inadequate Tfh differentiation and recruitment to GCs (Qi 2012, Qi et al 2008).

Following B:T conjugate formation, activated T cells migrate towards the interfollicular regions where they proliferate extensively (Coffey et al 2009, Cyster 2010, Vinuesa et al 2016) and by day 5 start migrating towards the centre of the follicle (Ansel et al 1999). T cell follicular entry is an active process, requiring a balance between CXCR5, CCR7, EBI2 and S1PR2 expression (Ansel et al 1999, Hardtke et al 2005, Haynes et al 2007, Moriyama et al 2014). As mentioned above, CXCR5 is expressed as early as 24hr after activation (Baumjohann et al 2011, Kitano et al 2011). This is followed by a sharp increase of CXCR5 expression in a BCL6-dependent manner reaching a plateau at day 3 (Liu et al 2012). At the same time CCR7 and EBI2 expression is downregulated by BCL6, promoting GC confinement (Hardtke et al 2005, Hatzi et al 2015, Haynes et al 2007, Suan et al 2015). CXCR5 knockout T cells or T cells that could not downregulate CCR7 could still localise at the T/B border and interfollicular regions but were excluded from the follicle (Hardtke et al 2005, Haynes et al 2007) highlighting the importance of this regulation for GC homing. Additionally, Tfh cells express high levels of S1PR2 (Moriyama et al 2014), which directs cells away from its ligand S1P found at the perimeter of the follicles, similarly to GC B cells (Green & Cyster 2012, Green et al 2011). S1PR2 promotes Tfh retention within the GC structure as two-photon imaging of transferred T cells in mouse LNs has shown that

S1PR2 knockout Tfh cells were more likely to exit the GC once they reached the GC border region compared to S1PR2-sufficient T cells which tended to return to the GC (Moriyama et al 2014). S1PR1 downregulation further inhibits Tfh attraction towards S1P at the follicular border and prevents GC egress (Hatzi et al 2015).

Both DCs and B cells act as potent antigen presenting cells (APCs) for T cells and deliver pMHCII, ICOSL, CD80/86, CD40 and OX40L, which interact with TCR, ICOS, CD28, CD40L and OX40 on T cells respectively (Vinuesa et al 2016). Additionally, IL-6 cytokine secretion from APCs promotes upregulation of BCL6 (Choi et al 2013), and therefore Tfh differentiation (Johnston et al 2009, Nurieva et al 2009, Yu et al 2009). BCL6 expression (Deenick et al 2010) is gradually upregulated from day 1 to 7 after activation (Baumjohann et al 2011, Liu et al 2012) and leads to commitment to the Tfh lineage by upregulating expression of effector molecules such as PD-1, CXCR5, CXCR4 and SAP (Poholek et al 2010, Yu et al 2009), and repressing differentiation into other effector subsets like Th1, Th2 and Th17 (Hatzi et al 2015, Nurieva et al 2009). Once Tfh cells are formed, their survival within GCs depends on continuous signals from GC B cells (Baumjohann et al 2013). Presentation of peptides on MHCII by GC B cells promotes more CD40L externalization by Tfh cells, enhancing CD40 signalling in GC B cells, which in turn increases ICOSL expression and leads to more efficient entanglement with Tfh cells (Liu et al 2015). Blocking this interaction by antibodies against CD40L or ICOSL abrogates the GC reaction (Choi et al 2011, Han et al 1995). Other key factors produced by Tfh cells include IL-21 and IL-4 which are important for GC B cells proliferation and upregulation of BCL6 (Gonzalez et al 2018). Additionally, autocrine signalling of IL-21 has a role in preventing Tfh apoptosis and promoting Tfh differentiation (Nurieva et al 2008, Vogelzang et al 2008)

as well as in inhibiting the development of T follicular regulatory (Tfr) cells. Thus, changes in IL-21 secretion by T cells can change the ratio between T follicular regulatory (Tfr) and Tfh populations (Ding et al 2014) potentially leading to suppression of the GC response (Ding et al 2014, Linterman et al 2011, Wollenberg et al 2011).

1.5.3 Role of dendritic cells in T cell priming

Conventional dendritic cells (cDCs) are the dominant antigen presenting cells that prime naïve T cells in lymphoid organs (Banchereau & Steinman 1998, Durai & Murphy 2016, Itano & Jenkins 2003, Meredith et al 2012). Highlighting the essential role of cDCs in activating T cells, in vivo priming of CD4+ T cells following immunisation with soluble antigen or in the context of bacterial infection was completely abrogated after the specific depletion of cDCs or by selectively inhibiting their ability to present antigen (Loschko et al 2016, Meredith et al 2012, Samstein et al 2013). cDCs can be subdivided into cDC1 and cDC2 subpopulations, based on their distinct surface marker expression, localisation and functions. cDC1-associated markers include CD8, XCR1 and CD205 and are dependent on the transcription factors Batf3 and IRF8. cDC2 cells express CD4, DCIR2 (33D1), CD11b and Sirpα and depend on the transcription factors RelB, Notch2 and Irf4 (Durai & Murphy 2016, Eisenbarth 2019). While both subsets can prime CD4+ and CD8+ T cells *in vitro* and *in vivo* (Calabro et al 2016b, Schnorrer et al 2006), *in vivo* cDC1 cells preferentially stimulate CD8+ T cells, whereas CD4+ T cell activation is primarily mediated by the cDC2 subset (Calabro et al 2016a, Dudziak et al 2007, Eisenbarth 2019). This differential dependency on cDC1 and cDC2 cells for T cell priming is in part due to their distinct localisation within the tissue, both before and after activation (Eisenbarth 2019). During steady state cDC1 cells can be found in the T zone and marginal zone,

while cDC2 cells are positioned mainly at the interfollicular regions (Eisenbarth 2019). After immunisation cDC1 and cDC2 cells migrate to non-overlapping regions of the T zone, with activated cDC1 cells clustering within the centre of the T zone where CD8+ T cells are most abundant, and cDC2 cells positioning at the CD4+ T cell-rich periphery of the T cell zone, in close proximity to the B cell follicles (Calabro et al 2016b).

Overall, the GC response is a highly complex process that depends on the spatiotemporal control of multiple cell types. While LNs and the spleen share many similarities in terms of cell composition and functions, there are also major organ-specific differences in the regulation of immune responses. Unique to the spleen, the marginal zone region is essential for the development of adaptive immunity. However, our understanding of the individual roles of MZ-resident cells in immunity, particularly that of MZ macrophages is limited.

1.6. Hypothesis

The presence of multiple subsets of macrophages in the splenic MZ suggests a high degree of functional specification. In this thesis, I tested the hypothesis that the SIGN-R1 expressing macrophage subset plays a specific and unique role in regulating GC B cell responses in the spleen.

1.7. Aims

1. To test whether specific depletion of SIGN-R1 macrophages has a role in GC regulation.

This aim utilized various approaches including the development of a new mouse model expressing the Cre-recombinase and Diphtheria Toxin Receptor (DTR) molecule under the SIGN-R1 locus, allowing the selective tracking and ablation of SIGN-R1-expressing macrophages.

2. To identify key mechanisms by which SIGN-R1 cells regulate the GC

This aim involved extensive analysis of the dynamic and architecture of the GC response in the spleen when SIGN-R1 macrophages are removed. Using a combination of approaches, we show that SIGN-R1+ macrophages are necessary for positional regulation of CD4+ DCs, a defect that is associated with a significant reduction in GC responses.

2 Materials and methods

Mice

C57BL/6 (B6, CD45.2+) and B6 Ly5.2 (CD45.1+) mice were purchased from Charles River Laboratories. MD4 (Goodnow et al 1988) (MGI: 2384162) and OTII (Barnden et al 1998) (MGI: 2174541), have been described previously. CD19 KO mice were in a B6 background and were generated by crossing CD19 Cre+/+ (Rickert et al 1995) mice to obtain CD19 Cre/Cre mice. Rosa26-stop-tdTomato (007914; B6.CgGt(ROSA)26Sortm14(CAG-tdTomato) were purchased from The Jackson Laboratory. Mice expressing DTR under the CD169 promotor (CD169-DTR mice) were described previously (Miyake et al 2007, Saito et al 2001) and purchased from the RIKEN BioResource Center. SIGN-R1-Cre/DTR mice were generated by Biocytogen using B6 ES cells. In these mice, a cDNA construct encoding an Frt-flanked neomycin resistance cassette followed by a stop cassette was introduced before a start codon, followed by cDNA encoding Cre recombinase, and the human DTR (HBEGF) was generated. The Cre recombinase and DTR coding sequences were separated by self-cleaving 2A viral sequences to allow stoichiometric translation of both individual proteins. This construct was inserted at the ATG start codon of cd209b (SIGN-R1), effectively replacing the expression of SIGN-R1. To remove the neomycin resistance gene and stop cassette, homozygous mice were crossed with B6.Cg-Tg(Pgk1-FLPo)10Sykr/J (The Jackson Laboratory), which expresses the mouse codon-optimized FLP recombinase under the direction of the mouse Pgk1 (phosphoglycerate kinase 1) promoter. Mice were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the Kennedy Institute of Rheumatology, University of

Oxford. All experiments were conducted in accordance with the UK Scientific Procedures Act (1986) under a project license authorized by the UK Home Office.

Macrophage depletion

CLLs or PBS-loaded control liposomes were purchased from Liposoma BV or Encapsula NanoSciences and were administered i.v. according to the manufacturer's instructions. To deplete macrophages in SIGN-R1-Cre/DTR mice, DT (Merck KGaA) was infused i.v. at 30 ng/g of body weight at 6, 4, and 1 days before immunization. The administration of DT was spread out over the course of 7 d before immunization to limit the effect of acute cell death of a large number of cells. We found that this DT administration schedule did not lead to any detectable inflammatory effects at the time of immunization. An additional DT injection was given at 3 d after immunization to ensure maintenance of SIGN-R1 macrophage depletion throughout the response.

Flow cytometry

Spleens were collected in RPMI 1640 (2%FBS, 0.01M HEPES), mashed through a 40um cell strainer and counted. $1x10^6$ cells of each sample was added to a 96-well plate, samples were washed once with FACS buffer (PBS, 1mM EDTA, 0.1% azide, 2% FBS) and stained in 25 ul antibody mix in FACS buffer for 20min on ice. Samples were washed and analysed on BD LSR Fortessa or BD LSR-II. Data was analysed using FlowJo software. The following antibodies were used: B220 (RA3-6B2), IgD (11-26c-2a), CD23 (B3B4), CD1d (1B1), CD21/35 (7E9), CD45.1 (A20), GL7, FAS (Jo2), Nur77 (12.4), CD86 (GL-1), CD69 (H1.2F3), CD83 (Michel-19), CD80 (16-10A1), Bcl6 (BCL-DWN), CD38 (90), CCR6 (140706), CD138 (281-2), CCR7 (4B12), and DCIR2 (33D1). For 33D1 staining, FACS buffer without EDTA was used. For DC staining, spleens were cut into ~2mm pieces and digested for 15 min at 37º C in digestion buffer (1mg/ml collagenase type IV, 20ug/ml DNase I in collection medium),

mashed through 100μm mesh and washed once before staining. Fixable Viability Dye eFluor 780 (eBioscience) was used to stain dead cells, and PE (Thermo Fisher Scientific) was used to label PE-specific GC B cells. A Foxp3 transcription factor staining buffer set (eBioscience) was used for transcription factor staining.

Immunization

Unless stated otherwise, immunizations were administered i.p. in a volume of 200 μL. For immunization with HEL-OVA and OVA, Ribi adjuvant (Sigma-Aldrich; S6322) was reconstituted in 1.1 mL of PBS and mixed 1:1 with 0.5 mg/mL protein in PBS. For immunization with PE (Thermo Fisher Scientific; P801) and NP-CGG (Biosearch Technologies; N-5055C), 50 μg of the antigen was mixed at a 1:1 ratio with alum adjuvant and rotated for 10 min before injection. For immunization with SRBCs, 1 mL of sterile SRBCs (Thermo Fisher Scientific; 12977755) were washed once with 50 mL of PBS and then reconstituted in 2 mL of PBS. For PKH26-SRBC immunization approximately 200 × 106 PKH26 conjugated SRBCs were injected i.v. at 3 h before analysis by flow cytometry, as described previously (Yi et al 2015). For immunization with VLPs, 25 μg of VLPs were injected i.p. Qβ-VLPs were generated by A. Cruz-Gomes from the Bachmann lab using a prokaryotic expression vector based on pGEM encoding the Qbeta coat protein (pQb10), transformed into Escherichia coli JM109 cells (Sigma-Aldrich) as previously described (Gomes et al 2017, Gomes et al 2019, Kozlovska et al 1993).

Conjugation of HEL-OVA, SRBC-HEL and SRBC-PKH26

For conjugation of HEL-OVA, equal volumes 20.5 mg/ml HEL (Sigma, cat. L6876-5G) and 4.5 mg/ml OVA (Sigma, cat. A5503-5G) in phosphate buffer (65mM pO42-, ph7.5) were mixed together and spun down to remove any insoluble material. 1.2 ml 0.085 % glutaraldehyde in phosphate buffer was added to every 3 ml protein mixture

and gently stirred at RT for 1hr, protected from light. The reaction mixture was spun down to remove any precipitate and was dialyzed against PBS at 4°C overnight before it was run on protein chromatography column using Sephadex(R) G-100 beads (Sigma, cat. GE17-0574-02) to separate conjugated HEL-OVA from unbound protein. Purified HEL-OVA was concentrated to 0.5mg/ml for use in experiments. For immunization with SRBC-HEL, 1 mL of sterile SRBCs were washed three times in PBS and once with 10 mL of conjugation buffer (0.35 M mannitol [Sigma-Aldrich; M4125] and 0.01 M NaCl) with the brake off. Cells were reconstituted in 0.9 mL of conjugation buffer and the mixed with 20 μg/mL HEL. The solution was mixed for 10 min on ice, followed by the addition of 10 mg of EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] (Thermo Fisher Scientific; 22980) to a final volume of 1 mL, followed by 30 min of rocking gently on ice. The cells were washed four times in PBS and resuspended in a final volume of 2 mL of PBS (Goodnow et al 1988). For immunization with VLPs, 25 μg of VLPs were injected i.p. Qbeta VLPs were generated using a prokaryotic expression vector based on pGEM encoding the Qbeta coat protein (pQb10), transformed into Escherichia coli JM109 cells (Sigma-Aldrich), as described previously (Cielens et al 2000). For immunization with SRBCs conjugated to PKH26, fresh SRBCs were conjugated to PKH26 (Sigma-Aldrich) according to the manufacturer's instructions with 10 μL of PKH26 dye (1 mM) per 1 mL of blood cells resuspended in 1 mL of conjugation buffer.

Generation and adoptive transfer of *in vitro*-induced GC B cells and *in vivo*-induced pre-GCs

To induce GC B cells *in vitro*, CD45.1+ MD4 B cells were grown on irradiated (60 Gy) 40LB cells supplemented with rIL-4 (1 ng/mL; eBioscience; 34-8041-85), as described previously (Nojima et al 2011). The 40LB cell line was a kind gift from Daisuke

Kitamura. Six days later, B cells were harvested and analysed by flow cytometry to confirm GC B cell phenotype (live, B220+IgDlowFAS+GL7+). Induced GC B cells (2 to 3 × 106) were subsequently transferred into CD45.2+ recipient hosts. To induce pre-GCs *in vivo*, CD45.2+ B6 mice were treated with CLL or PBS and 3 weeks later were co-transferred with 5 to 6 × 10^6 OTII T cells together with 5 to 6 × 10^6 GFP+ or 5 to 6 × 10^6 CD45.1+ MD4 B cells. The next day, the mice were immunized with HEL-OVA. On day 2 postimmunization, spleens were collected and enriched for B cells using the CD43 MicroBeads Kit (Miltenyi Biotec; 130-049-801) according to the manufacturer's protocol. The frequencies of GFP+ and CD45.1+ pre-GC B cells of each immunized mouse were analysed by flow cytometry (defined as live, B220+ CD38+ GL7+ CCR6+). Equal numbers (1.5 × 10^5) of GFP+ pre-GC B cells and CD45.1+ pre-GC B cells (derived from CLL- and PBS-treated mice, respectively) were mixed and analysed once more by flow cytometry to ensure a 1:1 ratio before being transferred into CD45.1+ B6 recipients.

Reconstitution of the MZ B cell compartment

To reconstitute the MZB cell compartment, total splenocytes from B6 mice (30 to 40 × 10^6) were transferred to CD19 KO recipient mice for 11 to 14 days before spleens were collected and analysed by histology and flow cytometry.

Histology staining and analysis

Spleens and LNs were embedded in optimum cutting temperature (OCT) compound (Agar Scientific; AGR1180) and snapfrozen in dry ice with 100% methanol, after which 7-μm sections were cryostat-cut and collected on SuperFrost Plus Adhesion slides (Thermo FisherScientific). Sections were dried at RT overnight, fixed in cold 100% acetone for 10 min and then allowed to dry for at least 1 hour. Before staining, slides were rehydrated for 10 min in PBS (for IF analysis) or Tris-buffered saline (for IHC

analysis) and blocked with normal mouse serum (2%) and normal rat serum (2%) for 20 min in a humidified chamber. Primary antibody staining was performed for 2 to 3 hours at RT, followed by secondary staining for 1 hour at RT. For IF analysis, slides were mounted with Fluoromount-G (Cambridge Bioscience). IF images were captured with an Olympus FV1200 confocal microscopy system. For IHC analysis, slides were mounted with Aqua-Mount (Thermo Fisher Scientific) and imaged with a Zeiss Axio Scope A1. The following antibodies were used: Armenian hamster SIGN-R1 (22D1; Bioscience), CD169-FITC (3D6.112; Bio-Rad), MARCO (ED31; Bio-Rad), GL7-FITC (BioLegend), GL7-bio (eBioscience), PNA-bio (Sigma-Aldrich), IgD-FITC (11-26c.2a; BioLegend), IgD-bio (11-26c; eBioscience), anti–hamster-APC (BioLegend), anti–hamster-HRP (The Jackson Laboratory), anti–hamster-AP (The Jackson Laboratory), anti–rat-Cy3 (Stratech), SA-Cy3 (Thermo Fisher Scientific), anti–FITC-A488 (The Jackson laboratory), anti–FITC-HRP (Roche Applied Science), and SA-AP (The Jackson Laboratory). SIGN-R1-Cre/DTR homozygous mice and CD169-DTR homozygous mice were used as negative controls to confirm antibody specificity of a-SIGN-R1 and a-CD169 antibodies respectively. For 33D1+ DC staining, sections were rehydrated in TBS, and incubated for 4 min with 0.05% H2O2 to inactivate endogenous peroxidase. Slides were incubated with 33D1-bio (Biolegend) and IgD-FITC O/N at 4°C and the 33D1 signal was amplified using TSA Biotin amplification kit (Akoya Biosciences Inc.), followed by secondary staining with SA-HRP (The Jackson Laboratory), and anti-FITC-AP (Sigma). For imaging of tdTomato in SIGN-R1-Cre/DTR tdTomato f/f mice, spleens were cut into quarters and incubated in 4% PFA in PBS for 2 hours at 4 °C, washed three times with cold PBS for 5 min, dehydrated in 30% sucrose overnight, and embedded in OCT compound. To analyze the relative frequency of SIGN-R1–expressing cells in the spleens of WT, CD19 KO, and CD19

KO mice reconstituted with MZ B cells, spleens were harvested and stained as above with anti–SIGN-R1 antibody. Large tiles of sections were imaged using confocal microscopy. Surfaces surrounding the SIGN-R1–positive cells in each section were created automatically using Imaris software. The total area covered by surfaces and the total area of the section (μm2) were calculated using Imaris, and the ratio between them was plotted.

RNA sequencing

B6 mice were treated with CLL or PBS-L and 2 weeks later were transferred with $1x10^6$ MD4 cells, followed by HEL-OVA immunisation one day later. $5x10^3$ Tfh cells gated as PD-1^+CXCR5^+CD4^+B220^- live single cells and $5x10^3$ naïve T cells gated as PD-1^- CXCR5^-CD4^+B220^- live single cells were sorted from total splenocytes 3 days after immunisation into 300μl RPMI 1640 supplemented with 10% FBS. RNA was isolated using RNeasy micro kit (Qiagen, cat.74034]) and sent for Smart-Seq2 RNA sequencing at the Wellcome Trust Centre for Human Genetics.

The DESeq2 (https://www.bioconductor.org/packages/devel/bioc/html/DESeq2.html) package was used to prepare normalised, variance-stabilised gene-expression values (from readcounts generated with Salmon (https://combine-lab.github.io/salmon/) for downstream analysis). This analysis was performed by Steve Sansom. The gene expression values were then normalized by calculating a Z-score value for each sample to generate heat map representations using Prism 8:

$$gene\ expression\ Z\ score_{sample} = \frac{gene\ expression_{sample} - mean_{gene}}{standard\ deviation_{gene}}$$

***In vivo* antibody treatments and production of anti-DEC205-OVA**

To induce temporal depletion of SIGN-R1, B6 mice received one i.v. injection of 100 μg of anti–SIGN-R1 antibody (22D1; Bio X Cell) or control hamster antibody (PIP; Bio X Cell). One day later, mice were co-transferred with MD4 B cells or OTII T cells,

followed by immunization with HEL-OVA. The generation of anti-DEC205-OVA conjugated antibody has been described previously (Boscardin et al 2006). In brief, HEK293T cells were grown in a 10-cm dish in DMEM supplemented with 10% FBS and 10 mM Hepes and then transfected with plasmids encoding the heavy and light chains of DEC205-Ova antibody using Lipofectamine 2000 (Thermo Fisher Scientific; 11668019). On days 1 and 4 after transfection, the medium was exchanged with fresh medium. On days 4 and 6, the supernatant was collected, spun to remove cell debris, and adjusted to pH 7.0. The antibody was purified using an HiTrap GHP column (Sigma-Aldrich; 29-0485-81) according to the manufacturer's instructions. The product size was confirmed by SDS/PAGE. Mice were infused i.v. with 10 μg of purified anti–DEC205-OVA per mouse.

Statistical analysis

Unless stated otherwise, P values were calculated with unpaired two-tailed Student's t test for two-group comparisons. Data are displayed as mean ± SD. A P value <0.05 was considered significant; and the following values were delineated: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical analysis was performed with GraphPad Prism 8.

3 SIGN-R1 macrophages are important for germinal centre development

3.1 Introduction

The spleen is the biggest lymphoid organ in the body and the last barrier against systemic pathogen dissemination. It is responsible for capturing and mounting an adaptive immune response against pathogens that have breached the mucosal barriers and have entered the circulation (Bronte & Pittet 2013, Kim 2010, Mebius & Kraal 2005). Similar to LNs, the spleen facilitates the development of germinal centre (GC) reactions in response to pathogens. This process is critical for the generation of high affinity long-term humoral immunity and represents the main mechanism by which most current vaccines operate (Pollard & Bijker 2020). While the GC development has been extensively studied, our knowledge remains incomplete. This is particularly true in the spleen, where a unique region, called the marginal zone (Fig. 1), plays an important role in immunity to blood-borne pathogens.

Two major subsets of resident macrophages associated with the MZ have been identified: MZ macrophages and metallophilic macrophages (Gordon et al 2014, Martinez-Pomares & Gordon 2012). While both subsets have been shown to play roles in the regulation of immune responses (Gordon et al 2014, Martinez-Pomares & Gordon 2012) and peripheral tolerance (Miyake et al 2007, Ravishankar et al 2014), the specific contributions of each macrophage population to these processes and the mechanisms involved are not well understood. As mentioned in the introduction, unlike the metallophilic macrophages that lie underneath the marginal sinuses and express high levels of the sialic acid-binding receptor CD169, MZ macrophages are located outside

the follicles in the MZ compartment itself and show low to undetectable CD169 expression (Gordon et al 2014). In addition, MZ macrophages can be distinguished from metallophilic macrophages by the expression of the scavenger receptor MARCO and the lectin binding receptor SIGN-R1, which are absent in metallophilic cells. Notably, while MZ macrophages are typically referred to as a single population of cells, closer examination shows that they are in fact heterogeneous, and that in addition to cells expressing both SIGN-R1 and MARCO on their surface, the outer rim of the MZ also contains a subset of macrophages that express the MARCO receptor but lacks SIGN-R1 (Gordon et al 2014, You et al 2011). Previous studies have found that these two cell subsets not only are phenotypically distinguishable, but also differ in their developmental requirements and kinetics (Chen et al 2005, Ito et al 1999, Takahashi et al 1994, van Rooijen et al 1989), suggesting that the MZ macrophage population contains at least two distinct cell types. However, whether these subsets play distinct roles in regulation of adaptive immune responses remains unclear. In this thesis we refer to SIGN-R1+MARCO+ cells as "SIGN-R1 macrophages" and SIGN-R1-MARCO+ cells as "MARCO macrophages".

Major challenges of studying the individual populations have been the lack of specific genetic systems, insufficient specific cell markers, and the challenges of isolating tissue-resident macrophages with enough purity in sufficient numbers for in vitro culture or transcriptional analysis (Gray et al 2012). In this chapter we used a previously described pharmacological approach to selectively remove SIGN-R1 macrophages and we develop a new genetic mouse model to specifically target and deplete SIGN-R1 cells but not MARCO macrophages in order to uncover their specific function in the context of immunisation.

3.2 Selective ablation of SIGN-R1 macrophages in clodronate liposome-treated mice leads to impaired germinal centre B cell response

To study the functions of SIGN-R1 cells, we first utilized a clodronate liposome (CLL) approach to deplete splenic macrophages. Clodronate liposomes are made of concentric phospholipid bilayer spheres that contain clodronate solution. CLL is preferentially taken up by phagocytic cells and the clodronate is gradually released after fusion of the liposomes with the cell's lysosomes, leading to cell death. (Van Rooijen & Sanders 1994). Within a day from injection, all resident macrophages from the spleen are depleted, including red pulp (RP) macrophages, SIGN-R1, CD169, and MARCO macrophages, as well as MZB cells and dendritic cells (Aoshi et al 2008, van Rooijen et al 1989). While the exact time frame for cell recovery varies between studies, the repopulation kinetics reported are similar. RP macrophages, DCs and MZ B cells are the first to recover between 1 and 2 weeks after CLL injection (Backer et al 2010, Buiting et al 1996, Nikbakht et al 2013, van Rooijen et al 1989), followed by CD169+ macrophages - between 2 and 3 weeks after injection (Buiting et al 1996, van Rooijen et al 1989). SIGN-R1+ cells start reconstituting last, taking more than 4 weeks to reach normal numbers (Buiting et al 1996, van Rooijen et al 1989) (Fig.1b). This differential kinetic of repopulation was confirmed by histology (Appendix Figure 1) and provides a model to study the effect of specific SIGN-R1 absence on immune responses.

We first utilised the delay in SIGN-R1 cell recovery to test the importance of these cells in germinal centre (GC) development. We immunising mice at 3 weeks post CLL or control liposome injection. To accurately follow the specific responses, we used the well-established MD4 and OTII mouse model system. In this system, adoptively transferred splenocytes from MD4 mice, a B-cell transgenic mouse line expressing

BCR specific for HEL antigen, were co-transferred into hosts along with splenocytes from OTII animals, expressing TCR with high affinity to ovalbumin (OVA) antigen. One day after co-transfer, mice were immunized i.p with conjugated HEL-OVA in Ribi adjuvant. SIGN-R1-depleted mice had significantly lower frequencies of germinal centre B cells after immunisation suggesting a role for these cells in GC development (Fig.3). Similar results were obtained when mice were immunized with other model antigens including sheep red blood cells, phycoerythrin (PE) and NP-OVA (data not shown).

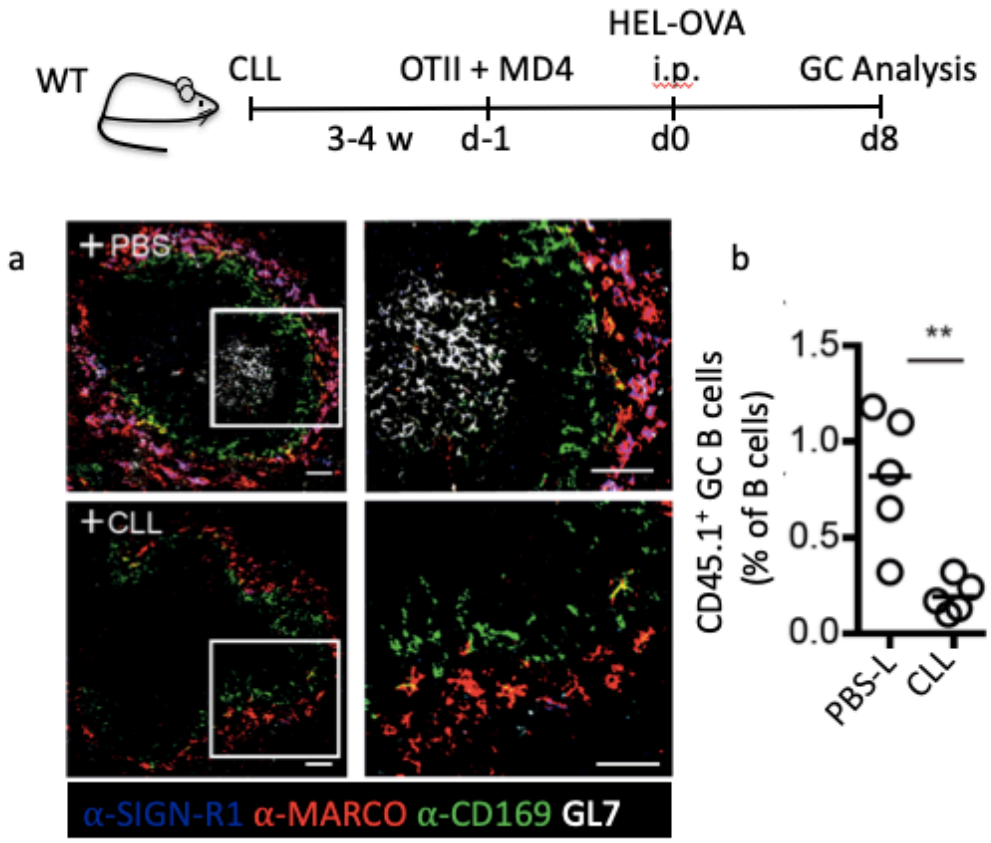


Figure 3. Depletion of SIGN-R1 macrophages impairs GC B cell responses

Top, experiment schematic. Clodronate-loaded liposomes (CLL) or PBS-loaded liposomes (PBS-L) were intravenously injected into CD45.2+ B6 recipient 3 weeks before co-transfer of OTII T cells and CD45.1+ MD4 B cells followed by HEL-OVA immunization. Spleens were analysed 8 days later. (a) Immunofluorescence analysis of immunized PBS-L- or CLL treated mice. White boxes identify the high magnification shown on the right. Scale 40 μm. Images were made by Anne

Chauveau. (b) Frequencies of HEL-specific GC B cells. Data represent one out of at least 5 independent experiments performed.

3.3 SIGN-R1 macrophages are dispensable for MZB cell localization

A key function of MZ macrophages is to capture and transfer opsonised antigens to marginal zone B cells (MZBs), which continuously shuttle between the MZ compartment and the B cell follicles, capturing antigens from the blood-exposed MZ compartment and transferring them to follicular dendritic cells inside the white pulp (Arnon et al 2013, Cinamon et al 2008, Cyster 2010). Disrupting this process could potentially cause inefficient presentation of antigen to cognate B cells leading to collapse of the GC response, which depend on antigen deposition on follicular dendritic cells (FDCs) (De Silva & Klein 2015). It was therefore possible that CLL treatment could alter the position of MZB cells and disturb this process. To test this, we used the previously described in vivo labelling approach to assess location of MZ B cells (Arnon et al 2013, Cinamon et al 2008). In this approach, fluorescently labelled antibodies are injected intravenously into the tail vein of mice. Five minutes later, the mice were scarified and the frequencies of labelled cells were determined by flow cytometry. Since the MZ is exposed to the open blood flow, cells located at the MZ are labelled, whereas those that are localized inside the follicles remain protected from labelling (Cinamon et al 2008). To test the positioning of MZB cells in the absence of SIGN-R1 macrophages we *in vivo* labelled PBS or CLL-treated mice with antibody against CD45, expressed by all hematopoietic cells including all B cells. As expected, the frequencies of $CD21^{hi}CD23^{low}$ MZBs were similar after CLL or control liposome injection indicating sufficient recovery of the MZB subset (Fig.4, bottom). Of these, around 50% were positive for in vivo labelled CD45 in both control and CLL-treated animals (Fig.3, top).

These results are consistent with the previously described distribution of MZB cells in wild type mice (Cinamon et al 2008), thus indicating SIGN-R1 cells are dispensable for MZB cell localization.

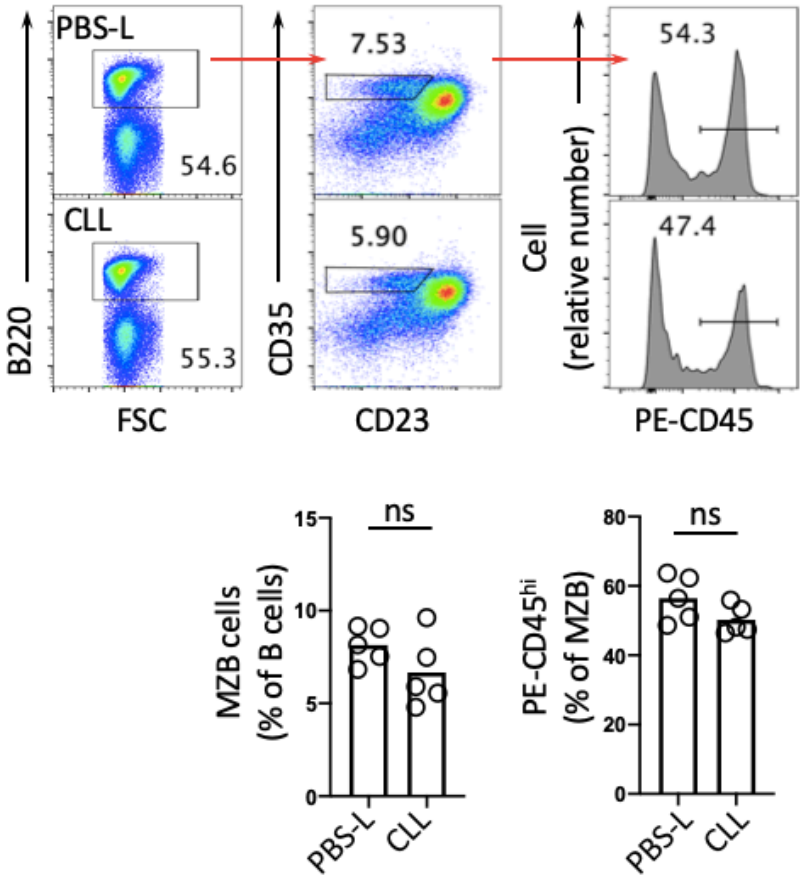


Figure 4. Frequencies and localization of MZB cells in SIGN-R1 macrophage-depleted mice are intact.

Mice that have been pre-treated with CLL or PBS-L 4 weeks earlier were in vivo labelled for 5 min with PE-CD45. Top, gating strategy to determine the frequencies of in vivo labelled (PE-CD45^{+}) MZB cells (B220^{+}CD23^{Low} CD35^{Hi}) in PBS-L and CLL mice, pre-gated on live, single cells. Bottom, quantification of MZB cell frequencies of B cells (left) and of in vivo labelled MZB cells (right). Figure shows one repeat of at least 2 experiments.

3.4 Depletion of the SIGN-R1 receptor does not affect GC B cell responses

SIGN-R1 is a C-type lectin receptor, homolog of the human DC-SIGN. Since previous studies showed that it can function as a pattern recognition receptor by interacting with certain pathogens and since some studies suggest it may play a role in fixing complement, we wondered whether loss of SIGN-R1 itself may have contributed to the impaired GC responses, possibly due to reduced antigenic and immune complex delivery into the follicles. To test this possibility, we removed SIGN-R1 from the cell surface before immunisation. We used the previously described monoclonal anti-SIGN-R1 antibody, 22D1, shown to transiently deplete SIGN-R1 from the cells without depleting the macrophages, an effect that lasts for up to 15 days after injection (Kang et al 2004). Mice were injected intravenously with 22D1 or control (anti-hamster) antibody. One day later mice were co-transferred with MD4 B cells, specific for HEL antigen, and OTII T cells, and immunised with HEL-OVA. Germinal center B cell responses were assessed by flow cytometry on day 8, while the absence of SIGN-R1 expression was confirmed by immunohistochemistry (Fig.5). GC frequencies were similar between SIGN-R1 depleted mice and controls. This data suggests that SIGN-R1 itself does not play an essential role in the development of GC responses to model antigens.

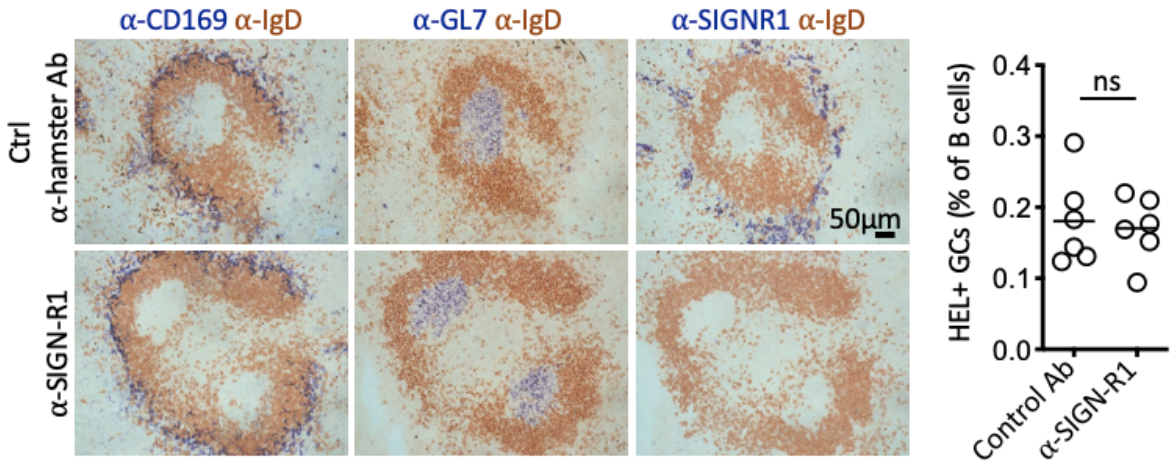


Figure 5. Temporal depletion of SIGN-R1 does not affect GC B cell responses.
Mice were injected intravenously with control anti hamster (Ctrl, upper panel) or hamster anti-SIGN-R1 (22D1, lower panel) antibody. One day later they were transferred with MD4 B cells and OTII T cells followed by HEL-OVA immunisation. Spleens were analysed 8 days later. Left, immunohistochemistry of consecutive sections from immunized mice stained for IgD (brown) and SIGN-R1, GL7 or CD169 (blue), as indicated. Sections from 3 (Ctrl) and 4 (22D1) treated mice were analysed in each experiment. Right, frequencies of HEL-binding GC B cells in the follicular compartment (defined as B220+IgDlowFAS+GL7+HEL+ live cells). Figure shows one representative example out of 3 independent experiments.

3.5 Development of a genetic model to selectively target SIGN-R1 cells

While the CLL treatment system is a robust and efficient system to approach the specific effects of SIGN-R1 cell depletion on GC development, it was still possible that later recovery of yet an unknown factor independently of SIGN-R1 cells may have contributed to the effect. We therefore wanted to confirm our findings with a more direct model using a genetic system. However, currently there are no genetic models to deplete SIGN-R1 cells specifically. To this end we designed a new genetic mouse model in which SIGN-R1 cells express Cre recombinase and diphtheria toxin receptor

(DTR) under the SIGN-R1 locus. The transgenic construct was designed to integrate just after the ATG start codon of exon 1 of the CD209b gene, resulting in the construct being expressed in place of the SIGN-R1 receptor. Mice homozygous for the transgene are therefore SIGN-R1 knockouts. The components of the transgene from 5' to 3' include a neomycin resistance gene (neo) and a stop cassette, both flanked by FRT sites, followed by codon-improved Cre recombinase (iCre) and DTR, separated by a 2A self-cleaving viral peptide. A BGH polyadenylation signal was added to terminate transcription and stabilize the mRNA (Fig.6a). Because of the stop cassette located upstream, Cre or DTR will not be expressed until FLP recombinase excises the FRT-flanked neo and stop cassette. We designed the construct this way to allow an additional level of specificity for future studies, if needed. We did this because wanted to have the flexibility of crossing the mice to a FLP-recombinase with a unique expression pattern such that in future studies we will be able to limit expression to an even more specific subset of SIGN-R1 expressing cells. However, in this study, we only wished to induce Cre and DTR expression under the SIGN-R1 locus. Therefore, to remove the stop cassette, we crossed the line to a delete stain mouse expressing FLP recombinase under the direction of the mouse Pgk1, phosphoglycerate kinase 1, promoter. In these mice high levels of FLP-recombinase are expressed ubiquitously from early gastrulation stages, thus allowing efficient deletion of FRT floxed cassettes. F1 of this breeding was crossed to itself and FLP was bred out in order to generate the final experimental line of SIGN-R1-Cre/DTR.

3.5.1 Validation of SIGNR1-Cre-DTR mouse model

To test if the Cre was selectively active in SIGN-R1 cells, we crossed SIGN-R1-Cre/DTR mice to Rosa26-stop-tdTomato mice. Spleens form SIGN-R1-Cre/DTR$^{+/-}$

tdTomato $^{+/-}$ mice were PFA fixed and snap-frozen and 7-um sections were stained and imaged for SIGN-R1 and Tomato by confocal microscopy. The tdTomato signal overlapped with SIGN-R1 expression (Fig.6d) suggesting correct expression of the construct. Additionally, SIGN-R1 cells in homozygous mice developed normally in the absence of the receptor, as evident by the presence of tdTomato+ cells in SIGN-R1-Cre/DTR$^{+/+}$ tdTomato $^{+/+}$ homozygous mice (Fig.6e). To test whether DT was active and expressed selectively in SIGN-R1 cells, we injected SIGN-R1-Cre/DTR mice with DT or PBS and stained spleen sections for SIGN-R1, MARCO and CD169 expression. We found that SIGN-R1 cells were depleted but CD169 or MARCO macrophages were unaffected, confirming a high level of specificity to SIGN-R1 cells (Fig.6b and 6c).

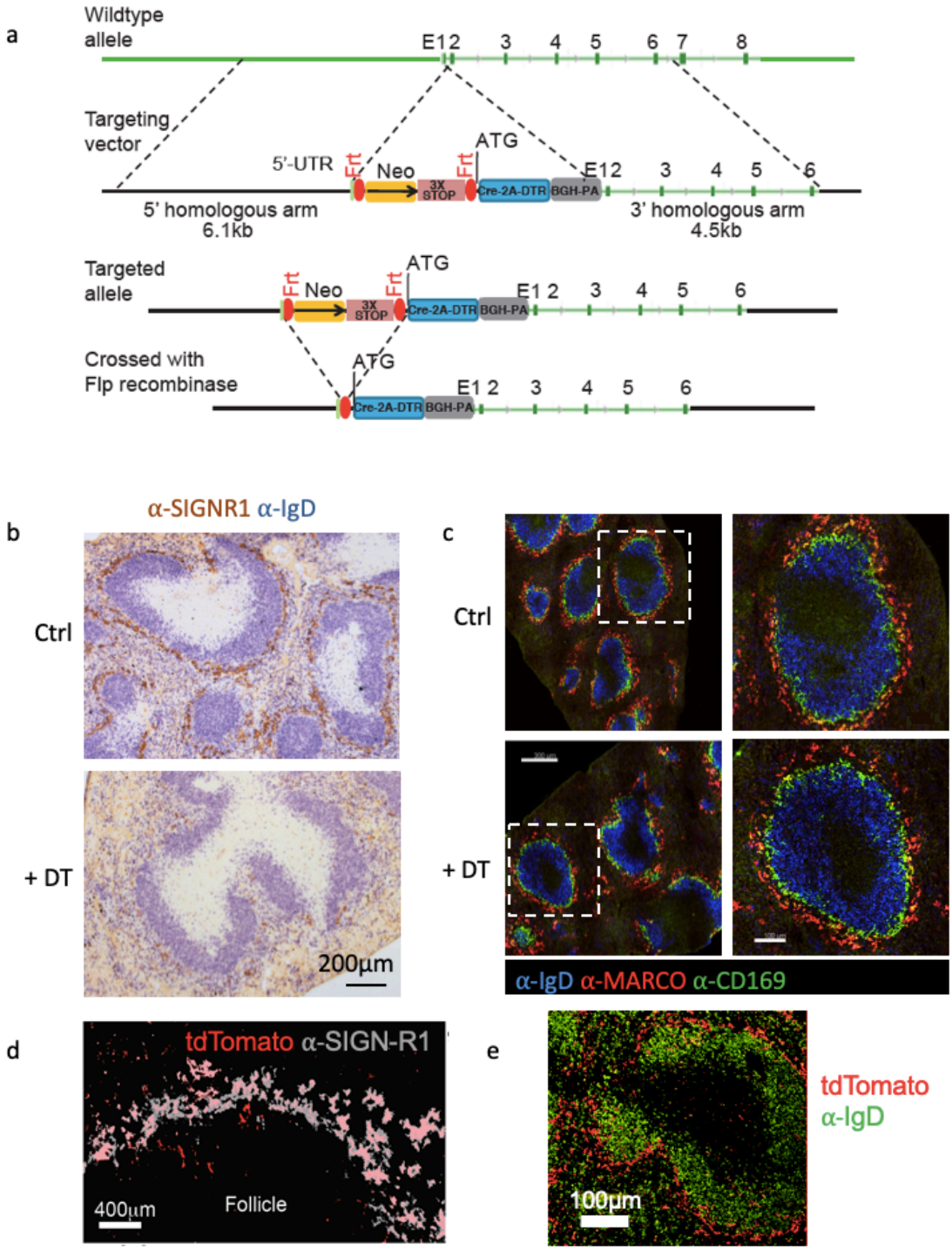


Figure 6. Validation of SIGN-R1-Cre/DTR mouse model.

(a) *Schematic of SIGN-R1-Cre/DTR mouse model design. The transgene was integrated after the ATG start codon of exon 1 of the SIGN-R1 gene, the targeting vector contains a neomycin resistance gene (neo) followed by a stop cassette, both flanked by Frt recombination sites, an iCre and DTR gene sequences separated by a 2A viral "self-cleaving" peptide sequence. A bovine growth hormone polyadenylation (bGHpA) transcription terminator was incorporated at the end of the construct. Mice*

homozygous for the transgenic allele were crossed with a line expressing FLP recombinase from the phosphoglycerate kinase 1 (Pgk1) ubiquitous promoter to excise the neo-stop cassette and allow expression of Cre and DTR. We refer to these mice as SIGN-R1-Cre/DTR. (b) SIGN-R1-Cre/DTR$^{+/-}$ heterozygous mice were injected with DT twice, 4 days and 1 day before spleens were collected to reduce the effect of macrophage death-related inflammation on the immunisation response (Chatziandreou et al 2017). Figure shows immunohistochemistry of spleen sections of a non-injected SIGN-R1-Cre/DTR$^{+/-}$ control (ctrl) and an injected mouse (DT) stained for SIGN-R1 (brown) and IgD (blue). (c) SIGN-R1-Cre/DTR$^{+/+}$ homozygous mice were injected with DT twice, 4 days and 1 day before spleens were collected. Immunofluorescence analysis of spleen sections stained for IgD (blue), MARCO (red), CD169 (green) White boxes identify the high magnification shown on the right. Scale, left panels - 300µm, right panels - 100µm. Two independent experiments, with at least two mice imaged per group. (d) Immunofluorescence of spleen section of SIGN-R1-Cre/DTR$^{+/-}$ tdTomato^{+} mouse showing co-localisation between SIGN-R1 (grey) and tdTomato (red). (e) Immunofluorescence of SIGN-R1-Cre/DTR$^{+/+}$ tdTomato$^{+/+}$ homozygous mice showing tdTomato (red) expression and IgD (green) stain. Sections are from at least 3 mice per group, 3 independent experiments

We also validated the transgene expression in LNs, where SIGN-R1 is expressed mainly in medullary macrophages and to a lesser extent in subcapsular sinus macrophages (Gray & Cyster 2012). By imaging the LNs of SIGN-R1-Cre/DTR$^{+/+}$ YFP$^{+/+}$ mice, we observed YFP^{+} cells were found mainly in the medullary compartment, matching the published localisation pattern of SIGN-R1 (Fig.7). DT administration efficiently depleted medullary macrophages but not CD169+ subcapsular sinus macrophages, further validating the specificity of our model for SIGN-R1 expressing cells (Fig.7). Similar observations were found in LN of SIGN-R1-Cre/DTR$^{+/-}$ heterozygous mice by IHC stain for SIGN-R1 and CD169 (data not shown).

Notably however, despite the above encouraging results, we found that in about 1/3 of the mice DT treatment led to a partial effect of incomplete ablation of SIGN-R1 cells. Possible explanation for this inefficiency includes random skipping of the translation of the gene that comes after the 2A cleavage site (Liu et al 2017) or the rate of SIGN-R1 gene transcription and translation could be lower than the turnover rate of DTR protein thus lowering the efficiency of the DT treatment. With this limitation in mind, in subsequent experiments each DT-treated SIGN-R1$^{Cre\text{-}DTR+/-}$ mouse was tested by histology to confirm efficient ablation of SIGN-R1 cells.

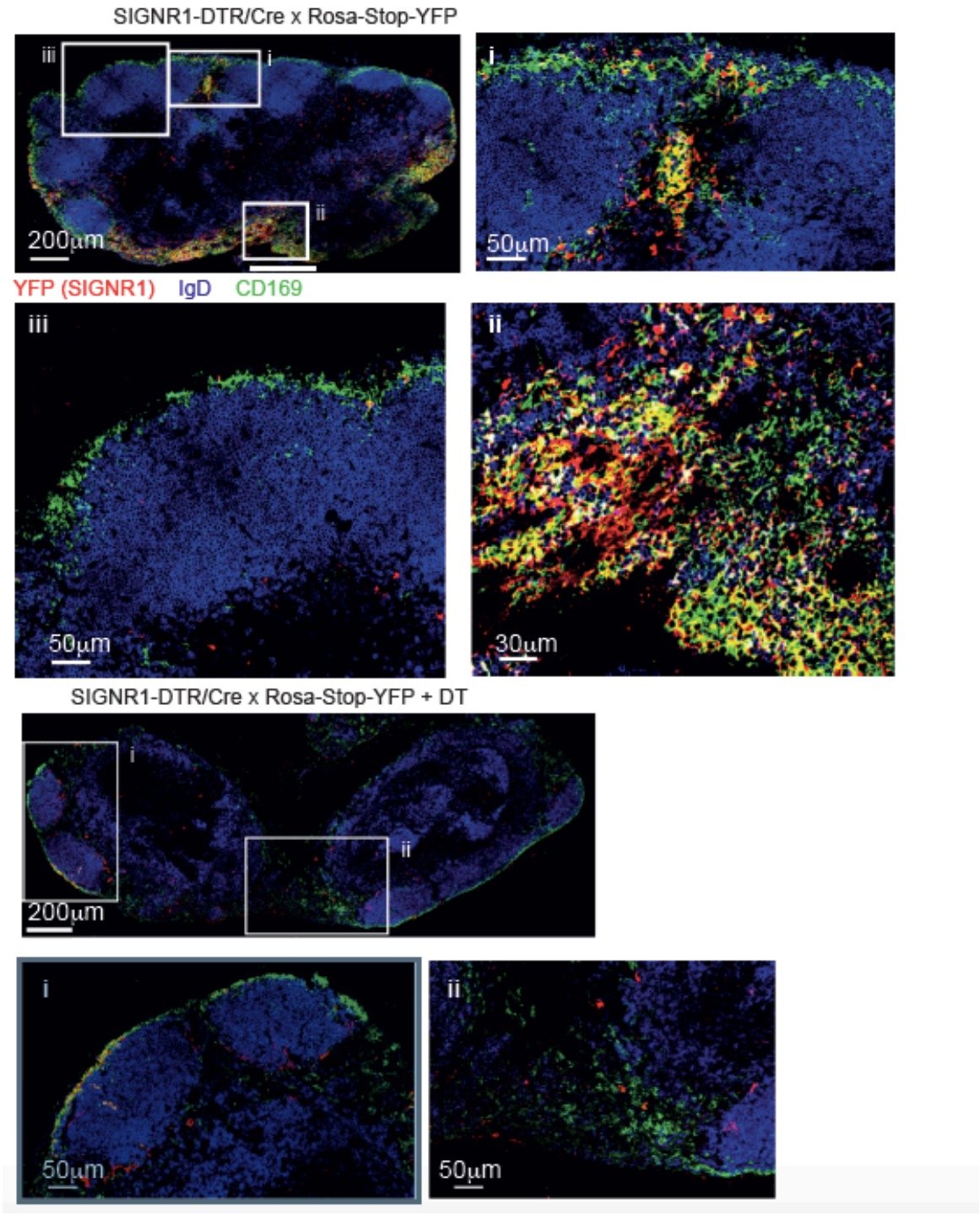


Figure 7. Selective ablation of SIGN-R1+ cells in lymph nodes of SIGN-R1-Cre/DTR mice *Immunofluorescence analysis of spleen sections of SIGN-R1-Cre/DTR+/- x R26-stop-YFP mice before (a) and after (b) DT treatment. Sections were stained for CD169 (green) and IgD (blue). Red, YFP signal. Boxed regions in top left images are shown in enlargements. Images represent one out of at least 3 mice tested.*

3.5.2 Selective depletion of SIGN-R1 cells in SIGNR1-Cre/DTR mice leads to impaired germinal centre B cell responses

To test the role of SIGN-R1 macrophages in regulating the GC response, SIGNR1-Cre/DTR heterozygous mice were injected with DT or PBS 6,4 and 1 days before intraperitoneal (i.p) immunization with PE (phycoerythrin) in alum, a model system that allows tracking of endogenous PE-specific B cells (Taylor et al 2012). We administered DT 6 days before immunization in order to minimize the inflammation associated with the initial DT-induced cell death at the time of immunization, while later injections were to ensure persistent macrophage depletion. Analysis of inflammatory cytokines (e.g. IL6, IL-10, IFN-γ) confirmed that this approach ensured resolution of the inflammatory response by day 0 (Appendix Figure 2). Seven-eight days post immunization, the spleens were harvested, halved and analysed by FACS and immunofluorescence. We found that when SIGN-R1 cells were depleted, the frequency of PE-specific GC B cells ($B220^{+}IgD^{low}FAS^{+}GL7^{+}$) was significantly reduced compared to control animals (Fig.8a). Immunofluorescent analysis further confirmed these results, showing loss of GL7+ GCs when SIGN-R1+ cells were selectively ablated (Fig.8b). These findings provide a direct and important support for the notion that SIGN-R1 macrophages play a specific and non-redundant role in GC responses.

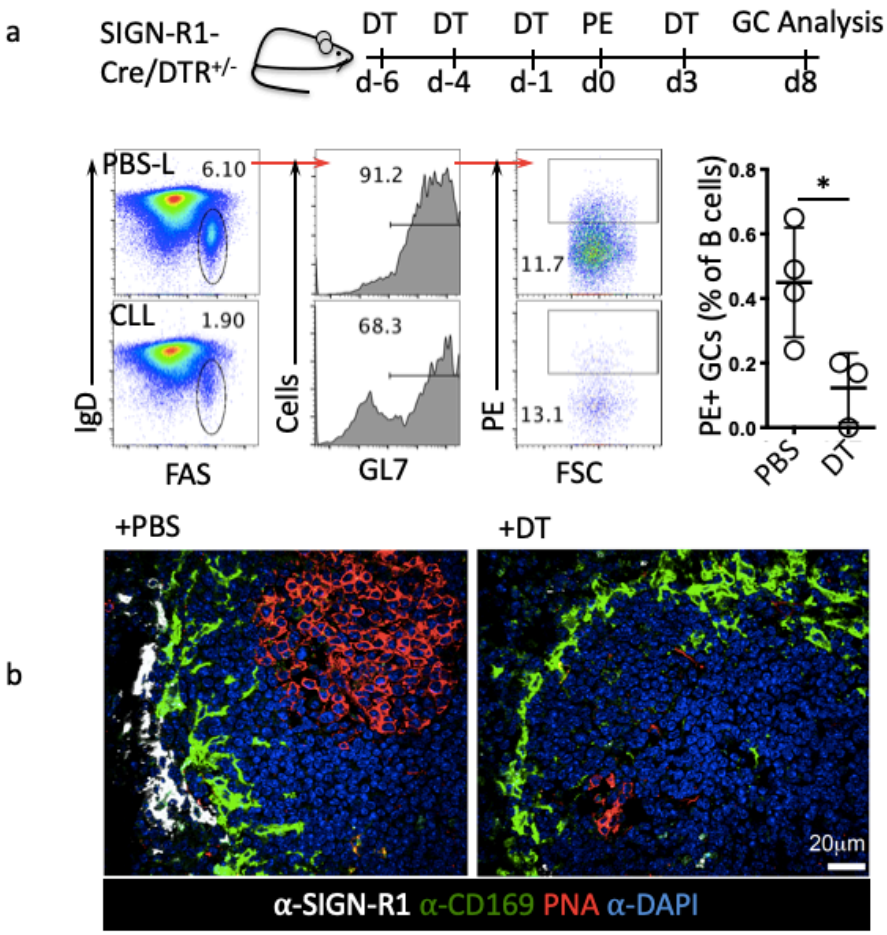


Figure 8. Specific SIGN-R1 depletion reduces the germinal centre response*. Top, schematic. SIGNR1-Cre/DTR heterozygous mice were treated with DT or PBS on days -6, -4, and -1 before immunization with PE/alum. One additional DT injection was given 3 days post immunization. Spleens were analyzed on day 8 by flow cytometry (a) and immunofluorescence (b). In 'a', left histograms show gating strategy to identify PE specific GC B cells. On the right, quantification of GC B cell frequencies. Graph shows representative data of 4 independent experiments performed. (**B**) Immunofluorescence staining of PBS and DT-treated mouse. SIGNR1 (white), CD169 (green), PNA (red), DAPI (blue). Scale 20um.*

3.6 Conclusions and discussion

In this chapter we tested the specific role of SIGN-R1 splenic macrophages in the development of GC responses. To address this question, we first used the clodronate

liposome model, in which the slow recovery rates of SIGN-R1 cells allows a window of time in which these cells are selectively missing. Under these conditions, we observed that in the absence of SIGN-R1 GC B cell frequencies were reduced. This effect was consistent for both transgenic and endogenous B cell responses, and across several immunisation systems, indicating a critical role for these cells in GC B cell responses in the spleen. This observation was further confirmed by a new genetic mouse model that we designed and validated, in which diphtheria toxin receptor and Cre recombinase are expressed under the SIGN-R1 locus, thus allowing specific depletion following DT injection. We also demonstrated that antibody-mediated depletion of the SING-R1 molecule from the cell surface has no detectable impact on GC formation against model antigens. Additionally, we showed that MZB cell positioning is normal in the absence of SIGN-R1 cells, excluding this as a potential pathway to explain the impaired GC phenotype observed in SIGN-R1 depleted mice.

Our data is in agreement with a previous study where clodronate liposomes treatment was used to explore the relevance of splenic macrophages to B cell responses in the spleen. In that study, the authors found that GC development was defective after short-term CLL treatment, which depleted SIGN-R1, MARCO and CD169 macrophages (Nikbakht et al 2013). However, they did not specify which of the three known subpopulations are necessary for the response and they did not explore the mechanisms involved. By using a specific genetic model for SIGN-R1 depletion, we were able to reveal a specific function of SIGN-R1 cells in GC formation. These findings are surprising because SIGN-R1 macrophages have been predominantly known for their innate immunity role in capturing pathogens, apoptotic cells or fixing complement. (Kang et al 2006, Kang et al 2004, Koppel et al 2005, Lanoue et al 2004). Furthermore,

SIGNR-1 cells remain sessile within the MZ and do not migrate during GC formation, suggesting their effect on GC responses is indirect. However, SIGN-R1 cells have been implicated before in indirectly modulating the immune response. In studies of mouse models designed to mimic the therapeutic effect of purified monomeric IgG in the treatment of autoimmune diseases in humans, SIGN-R1 was identified as the receptor important for binding the sialylated Fc part of IgG. This was suggested to induce secretion of soluble anti-inflammatory factors by SIGN-R1 cells that subsequently reduced the inflammation in an induced arthritis model (Anthony et al 2008). This demonstrates the pleiotropic roles of SIGN-R1 cells in immunity.

The generation of SIGN-R1-Cre/DTR mice provides a unique tool to further explore this cell subset allowing for their specific genetic modification. Here we used a ubiquitously expressed FLP to excise the stop cassette from the transgene in the whole genome. In the spleen and LNs, we find that Cre activity, visualised by Tomato labelling, correlates well with SIGN-R1+ cells. We also validated the capacity of DTR to specifically ablate SIGN-R1 cells in spleen and LNs within a day after DT injection. This data suggests that the model we have generated is a valid and useful tool for studying the effects of specific SIGN-R1 depletion as well as specifically modifying this subset. Despite the good overlap between SIGN-R1 and tdTomato in SIGN-R1-Cre/DTR$^{+/-}$ tdTomato^{+} mice, we have noted the occasional Tomato^{+} SIGN-R1^{-} single labelled cells found in the MZ suggesting some SIGN-R1 cells downregulated the receptor. This may reflect cells that have upregulated SIGN-R1 earlier during development or a low level of leakiness of Cre expression. In future studies, a more specific FLP could potentially further narrow down Cre and DTR expression.

As mentioned in the introduction, SIGN-R1+ cells are also observed in LNs, suggesting these cells could have similar function in GC development. While LN-resident SIGN-R1cells, similarly to the spleen, are positive for MARCO, they are also high in CD169 and F4/80 markers (Gray & Cyster 2012). Additionally, since there are no MZB cells in LNs, LN-resident SIGN-R1+ cells must have unique survival requirements. Apart from the phenotypic distinction, there is also a difference in the positioning of LN-resident SIGN-R1 cells relative to other lymphoid populations, which could result in LN-specific cellular interactions and functions - SIGN-R1+ macrophages do not encircle B cell follicles and are not positioned at the site of antigen entry as in the spleen. Instead, they are located within the LN medulla, a site where lymph and lymphocytes egress (Gray & Cyster 2012) (Gonzalez et al 2010). LN SIGN-R1 macrophages are also efficient at capturing a variety of antigens (Gray & Cyster 2012) (Gonzalez et al 2010), with the SIGN-R1 receptor itself shown to stimulate deposition of influenza antigen on DCs in the LN medulla (Gonzalez et al 2010). However, interrupting this interaction by blocking the SIGN-R1 receptor had no consequence on the virus-specific antibody production (Gonzalez et al 2010), suggesting this pathway is redundant for the development of LN humoral responses in this system. In support of this observation, total macrophage depletion did not perturb antibody responses in LNs (Gonzalez et al 2010), but led to the systemic spread of antigen (Gonzalez et al 2010), suggesting the primary role of LN SIGN-R1 macrophages is to contain pathogens. However, overwhelming antigen spread following total macrophage depletion in the lymph node could mask any specific functions of LN-resident macrophages. Therefore, the current evidence does not definitively exclude a role for LN-resident SIGN-R1+ cells in adaptive immunity and future studies could use the SIGN-R1-Cre/DTR mouse model to specifically deplete the subset and study their specific functions in LNs.

4 SIGN-R1 macrophages are required for the maintenance, but not the initiation of the GC B cell response

4.1 Introduction

Optimal GC development requires the time-specific migration of activated B cells between different microenvironments where they receive appropriate activation and survival signals (Cyster 2010, Gatto & Brink 2013). This tightly regulated series of migratory events is essential for the sequential delivery of activation signals and subsequently for the optimal development of the adaptive immune response. Early after activation, B cells migrate into the perimeter of the follicles where they engage in a proliferation process lasting 2-3 days (Gatto et al 2009, Pereira et al 2009). During this time, the cells begin to upregulate a set of activation and chemotactic proteins, eventually leading to their entry into the centre of the follicle, where they establish the nascent GC (Fig.2). Because SIGN-R1 cells are positioned in proximity to the outer follicles and the bridging channels, one possibility we considered was that these cells may be involved in the regulation of this migratory step. In this chapter we test this hypothesis and assess whether removal of SIGN-R1 macrophages affects B cell positioning and initial activation.

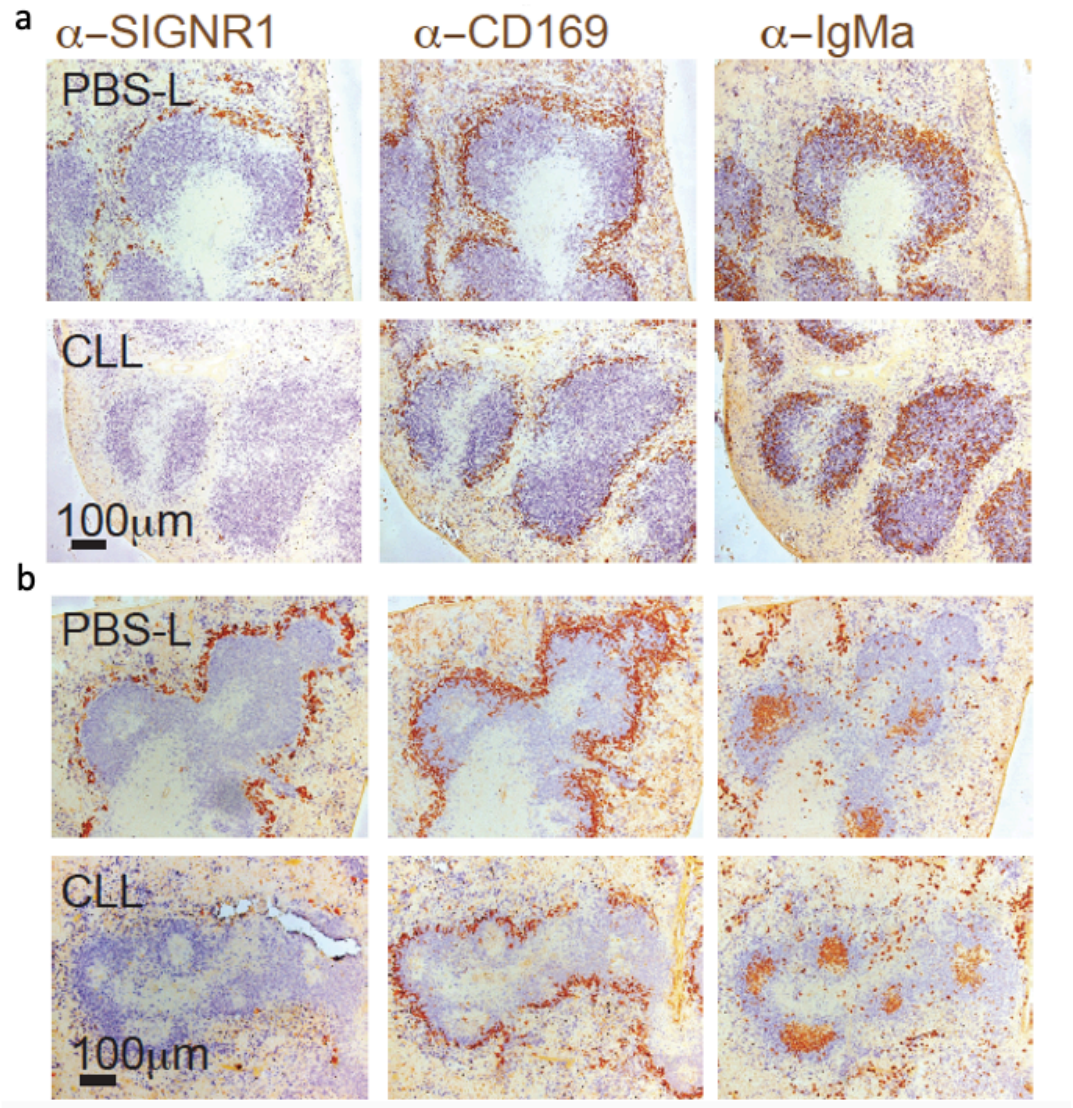


Figure 9. SIGN-R1 cells are dispensable for positioning of pre-GCs and early GC B cells

Distribution of HEL specific activated B cells. CD45.2+ B6 mice were treated with PBS-L or CLL and 3 weeks later were co-transferred with HEL-specific CD45.1+ MD4 B cells and OTII T cells followed by intraperitoneal immunization with HEL-OVA. Spleens were collected and snap frozen on day 2 (a) or 5 (b) after immunization. The distribution of activated B cells was assessed by immunohistochemistry staining for IgD (to highlight naïve endogenous B cells, blue) and IgMa (to identify transferred activated MD4 B cells, brown). Selective ablation of SIGN-R1 macrophages, but not CD169+ cells was confirmed by staining consecutive sections for IgD (blue) and SIGN-R1 or CD169 (brown). Data represent one example out of 3 independent experiments performed. In each experiment, sections from at least 3 spleens per group were analysed.

4.2 SIGN-R1 cells are dispensable for positioning of pre-GCs and early GC B cells

To test the relevance of SIGN-R1 cells in supporting the early step of migration towards the interfollicular regions, we tracked the movement of antigen-specific B cells in SIGN-R1-sufficient or depleted spleens. Mice that were treated with CLL or PBS-L 3 weeks earlier, were co-transferred with MD4 and OTII splenocytes and immunised with HEL-OVA antigen. The synchronous migration of the transferred B cells was tracked by the expression of IgMa by MD4 cells using histological analysis. Immunohistochemistry of consecutive sections confirmed selective loss of SIGN-R1 expression in mice treated with CLL (Fig.9a). Visualization of IgMa expressing cells in these spleens revealed no defects in the positioning of antigen specific B cells in the absence of SING-R1 macrophages. In both control and CLL-treated hosts, 2 days after immunization large numbers of antigen specific B cells were found in the perimeter of the FO (Fig.9a). This indicates that SIGN-R1 macrophages are not required for B cell migration to the follicular periphery and are not essential for maintaining the EBI2-ligand gradient in this region. Similar results were observed when both SIGN-R1, MARCO and CD169+ cells were ablated following a shorter course of CLL treatment (Fig.10).

However, it remained possible that SIGN-R1 cells could potentially be involved in the downregulation of EBI2 receptor on activated B cells, which is required for their migration towards the centre of the follicle and the subsequent initiation of the nascent GC reaction. To test this, we looked at the positioning of activated B cells 5 days post immunization. We found that similarly to the control group, at this time point activated B cells in CLL treated mice were able to leave the follicular perimeter and concentrate in the centre of the follicle (Fig.9b).

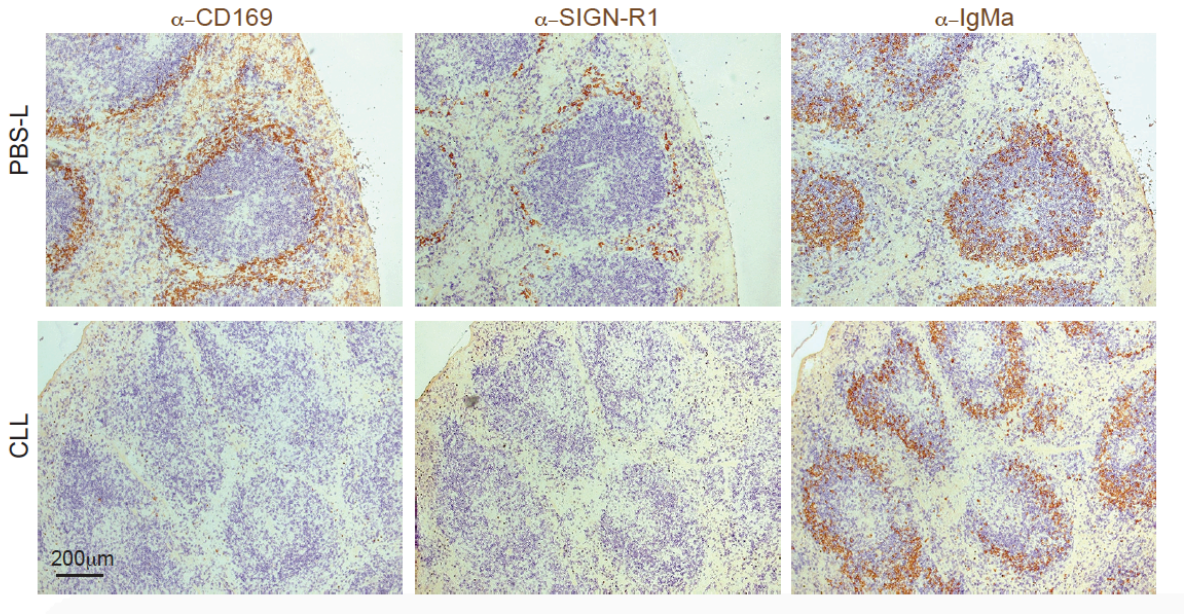


Figure 10. Positioning of activated B cells in the spleens of MZ and metallophilic macrophage-depleted mice. *MD4 and OTII cells were co-transferred into recipient mice that were treated with CLL or PBS-L 6 days earlier. The next day, mice were immunized with HEL-OVA. Two days post immunization, spleens were frozen and the distribution of activated MD4 B cells was assessed by immunohistochemistry staining for IgD (naïve endogenous B cells, blue) and IgMa (to identify transferred MD4 B cells, brown). Selective ablation of SIGN-R1 and CD169+ macrophages was confirmed by staining consecutive sections for IgD (blue) and SIGN-R1 or CD169 (brown). Data represent one example out of 4 experiments performed. In each experiment, sections from at least 3 spleens per group were analysed.*

4.3 Initial activation of B cell in the absence of SIGN-R1 cells is intact

The above data shows that SIGN-R1 macrophages were dispensable for the sequential migration of newly activated B cells and that under these conditions, the cells were able to localize to the follicular perimeter, where initial proliferation and acquisition of pre-GC phenotype has been proposed to occur (Coffey et al 2009, Taylor et al 2012). These pre-GCs are known to express high levels of CD38, GL7 and CCR6 and have the potential to differentiate into $CD38^{+}GL7^{-}$ memory B cells or to downregulate CCR6, upregulate BCL6, and commit to the GC pathway (Taylor et al 2012). To test whether

SIGN-R1 cells were important for pre-GC development, we first assessed the frequencies of $CD38^{+}GL7^{+}CCR6^{+}$ pre-GCs in SIGN-R1-deficient or sufficient mice. We co-transferred MD4 and OTII cells into PBS-L or CLL-treated animals and immunised them with HEL-OVA. The phenotype of the cells was analysed by flow cytometry at day 2 after immunisation. We found no significant differences in pre-GC frequencies of B cells between the two groups (Fig.11a,b).

Next, to test whether these pre-GCs are functionally intact, we compared the capacity of B cells that were primed in SIGN-R1 macrophage-depleted or sufficient hosts to become mature GC B cells. To do this, control and CLL-treated animals received congenically marked HEL-specific MD4 B cells (CD45.1+ and GFP, respectively) together with OTII cells followed by immunization with HEL-OVA. Two days later spleens were collected, enriched for B cells and analysed by flow cytometry to determine the frequencies of pre-GCs. We then co-transferred the same number of pre-GCs from CLL and PBS-L into WT CD45.2+ hosts (Fig.11c). As expected from our observations above (Fig. 11a,b), the frequencies of pre-GC B cells derived from SIGN-R1-depleted and sufficient mice were similar such that the ratio between GFP+ and CD45.1+ pre-GC B cells in the mixture was also close to 1:1 (Fig.11d). In order to allow transferred pre-GC B cells to be incorporated into existing GC structures and to provide a synchronized T cell help, the WT hosts were immunized with SRBCs 2 days earlier. One day after the transfer, recipient mice were boosted with SRBCs conjugated to HEL. Thus, in this system, endogenous SRBC-specific Tfh cells provide help to transferred HEL-specific B cells. 8 days after the boost we analysed the frequencies of MD4 GC B cells by flow cytometry. This analysis revealed similar frequencies of GFP+ (CLL derived) and CD45.1+ (PBS-L derived) GC cells suggesting priming in a SIGN-

R1 macrophage-depleted environment did not compromise the ability of B cells to mature into GC cells in a normal environment (Fig.11e). In support of this data, GC B cells induced *in vitro* by incubation with IL-21 and the 40LB stromal cell line (Nojima et al 2011), failed to develop into mature GCs B cell upon transfer to macrophage-depleted mice, with significantly lower GC frequencies compared to cells recovered from macrophage-sufficient hosts (Appendix Figure 3). Together, this data suggest that the early positioning and activation of B cells is largely independent of SIGN-R1 macrophages.

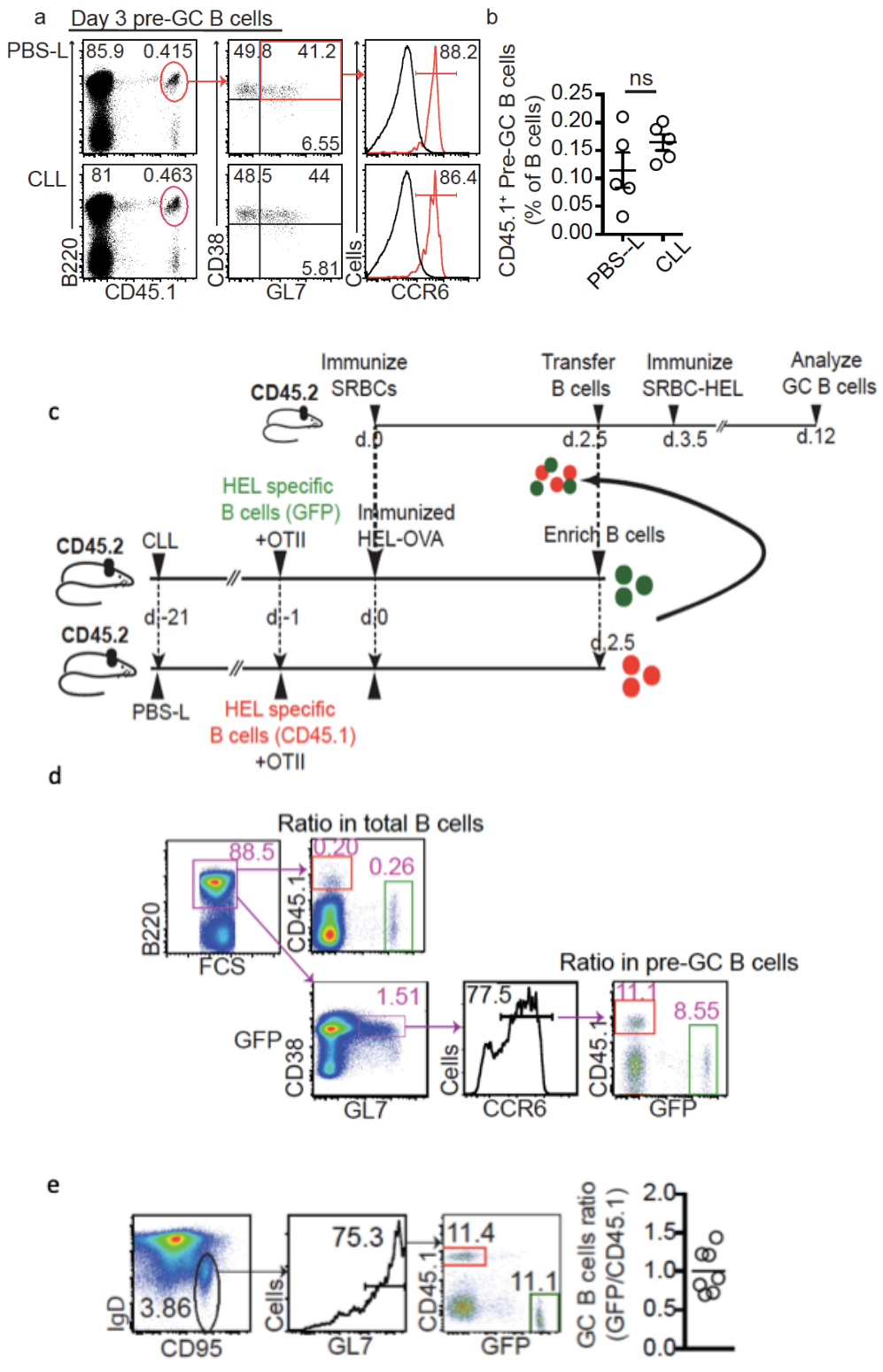


Figure 11. Pre-GCs are numerically and functionally normal in the absence of SIGN-R1 cells. *(a,b) CLL or PBS-L were injected intravenously and 3 weeks later MD4 (CD45.1$^+$) and OTII cells were co-transferred followed by intraperitoneal immunization with HEL-OVA. Spleens were analysed 3 days post immunization. (a) Representative flow cytometry plots to identify pre-GC B cells. Histograms show CCR6*

expression in pre-GC B cell (red) with naïve B cells (grey) as a negative control (gated on $B220^+$ $GL7^-$ $CD45.2^+$). (b) Percentage of $CD45.1^+$ specific pre-GC B cells in the B cell compartment. Figure shows a representative example out of at least 4 independent experiments performed. (c) Top, graphic representation of the experimental design. (d) Flow cytometry plots showing gating strategy to determine the ratio between B cells derived from PBS-L (CD45.1+) and CLL (GFP+) treated donors before transfer to SRBC-immunized mice. Top panel shows ratio of CD45.1/GFP cells in the total B cell compartment. Lower panel shows the ratio in the pre-GC B cell compartment, gated as shown. (e) Flow cytometry plots showing gating strategy to determine the relative frequencies of transferred $CD45.1^+$ and GFP^+ cells within the GC compartment recovered from recipient mice. Plots were pre-gated on $B220^+$ live cells. Right, data pooled from 3 independent experiments showing the ratio between $CD45.1^+/GFP^+$ cells within the GC compartment after transfer.

4.4 Kinetic and phenotype of early GC B cells

The results above suggest that GC initiation is independent of SIGN-R1 macrophages. To better define the stage at which SIGN-R1 macrophages are required, we looked at the kinetic of the response in the presence or absence of SIGN-R1 macrophages. We immunised CLL or PBS-L-treated animals with SRBC and assessed the frequencies of GCs at day 5, 10 or 15 after immunisation.

Four-five days post immunization, frequencies of GC B cells were similar in control and SIGN-R1 macrophage-depleted animals, supporting the notion that early priming is intact (Fig.12a). Phenotypically, we could not identify any abnormalities in these cells, as many of the typical markers that are upregulated in GC B cells at this stage were expressed at normal levels early after activation, including Nur77, a sensitive reporter of BCR stimulation (Mittelstadt & DeFranco 1993, Zikherman et al 2012), BCL6, the master regulator of GC B cell differentiation, as well as the activation markers CD80, CD83, CD86, and CD69 (Victora & Nussenzweig 2012) (Fig.12b). This

data is consistent with the notion that SIGN-R1 macrophages are dispensable for the initiation of GC B cell response in the spleen, but are necessary for its maintenance.

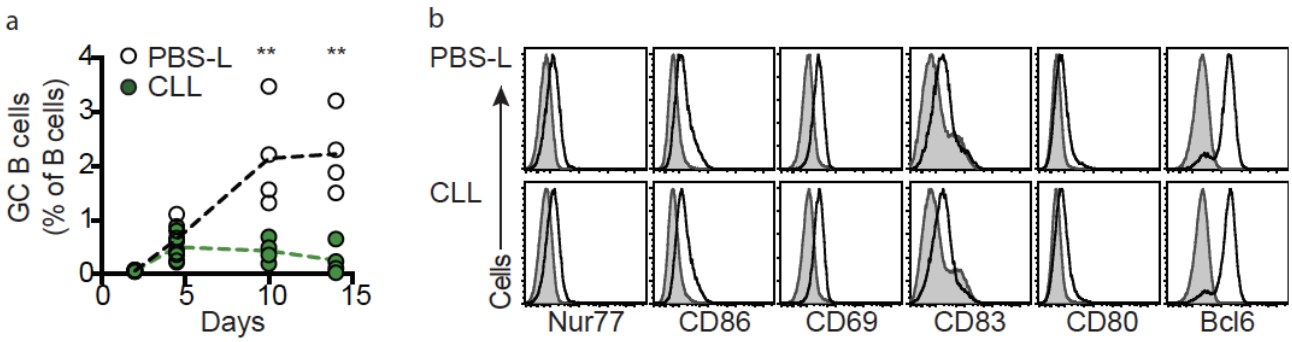


Fig.12 Kinetic and phenotype of early GCs in the absence of SIGN-R1 cells

(a) B6 mice were injected with CLL or PBS-L 3 weeks prior to intraperitoneal immunization with SRBCs. Spleens were analysed in the indicated time points by flow cytometry to determine frequencies of GC B cells ($B220^{+}IgD^{low}CD95^{+}GL7^{+}$ live cells) amongst total B cells. The experiment represents one out of two performed (n=> 3). (b) Flow cytometry analysis of GC B cells from day 5 after SRBC immunization of mice treated with PBS-L (top panel) or CLL (lower panel) as above (n=3). Grey histograms show stain in naïve B cells ($B220^{+}IgD^{high}CD95^{-}GL7^{-}$) in immunized animals. Figure shows one representative example out of 3 independent experiments performed.

4.5 Conclusions and discussion

In this chapter we find that SIGN-R1 macrophages are dispensable for the initial activation of cognate B cell and for their positional regulation. We showed that antigen-specific B cells were able to localise to the periphery of B cell follicles as well as to the follicular centre in the absence of SIGN-R1 macrophages. Flow cytometry analysis of activated B cells at different time points after immunisation revealed no differences in the frequencies of pre-GCs at day 2 or early GC cells at day 5 after immunisation. Instead, the defects we observed when SIGN-R1 cells were depleted were only evident at the mature GC stages indicating inability to sustain, rather than induce the GC

response. This notion is supported by the fact that we could not detect phenotypic or functional defects in pre-GCs that were induced in SIGN-R1 macrophage-depleted environment. Under these conditions the cells upregulated key activations markers similarly to cells primed in macrophage-sufficient hosts and were able to develop with similar efficiency into GC B cells when transferred into WT recipients. This further suggests that the GC defect in the absence of macrophages is unlikely to relate intrinsic suboptimal activation of B cells, but rather to impair the ability to maintain the mature reaction.

Our findings are consistent with a previous study in which GC development was shown to be abrogated following acute depletion of all splenic macrophages after CLL (Nikbakht et al 2013). However, contrary to our findings, in that earlier report, failure of CLL treated mice to develop GC B cells was attributed to inability of antigen-specific B cells to migrate to the perimeter of the splenic follicles (Nikbakht et al 2013). These observations led the authors to propose a role for MZ macrophages in regulating migration of activated B cells. It is possible that the discrepancy between the studies is related to the manner by which CLL was administrated - while in our studies we waited at least two weeks before immunization, in the previously published work mice were immunized 2-4 days post CLL injection. Since CLL treatment causes short term global changes in the architecture of the tissue, temporary loss of lymphoid stromal cells which are the main source of Ebi2 ligand necessary for migration to the follicular perimeter (Gatto and Brink, 2013) may have contributed to the impaired B cell migration reported previously. Consistent with this notion, previous studies have demonstrated that macrophages in the MZ are unlikely to be the source of 7a,25OHC, the Ebi2 ligand that mediates activated B cell migration to the perimeter of follicles (Yi et al 2012). In this

paper, Yi et al reconstituted CD169-DTR mice with bone marrow from CH25H-deficient animals. Because CH25-H is a key enzyme required for the generation of the Ebi2 ligand 7a,25OHC, cells that are derived from the donor BM are unable to produce it and therefore cannot provide the chemotactic gradient needed to localize activated B cells to the follicular perimeter. Of note, the authors treated the chimeras with DT, to ablate all WT host CD169+ cells (which indeed may be partially irradiation resistant), such that any newly generated CD169+ macrophages originated from the donor BM will be CH25H-deficient and therefore unable to synthesize 7a,25OHC. Under these conditions, activated B cells were found to localized normally at the follicular perimeter, strongly suggesting that CD169+ macrophages are not required for EBI2 ligand synthesis and are not necessary for directed movement of activated B cells to these follicular perimeter sites (Yi et al 2012). This conclusion could be extended towards SIGN-R1 macrophages as they express some CD169-DTR mRNA and are efficiently depleted after DT (Miyake et al 2007, Ravishankar et al 2012). Notably, the failure of MZ macrophage-depleted animals to form GCs we and others observed significantly exceeds the defects associated with EBi2 deficiency in B cells (Gatto et al 2009, Pereira et al 2009), further supporting the possibility that additional mechanisms are involved.

The organization of the splenic MZ is somewhat reminiscent of the subcapsular sinus region in peripheral LNs, where newly arriving antigens are initially captured. In these sites, subcapsular macrophages bind immune complexes and transfer them to follicular B cells that then relay them to follicular dendritic cells. This process enhances activation of B cells at the early stages of the response and may contribute to optimal selection in the GC, but it is not needed for the maturation of GC B cells responses

(Carrasco & Batista 2007, Junt et al 2007, Phan et al 2007). In contrast, our findings suggest that in the spleen, macrophages at the portal of antigen entry are dispensable for the initial activation of B cells and that their role in regulation of B cell differentiation is more relevant to the later stages of the response, at a time when the GC reaction begins to mature. It is not clear why MZ macrophages differ from their LN counterparts and are not necessary for initial B cell activation. However, it is worth mentioning that one fundamental difference between the spleen and LNs is that in the latter, circulating cells and lymph-born antigens enter the tissue via different routes and their first encounter is in the follicles, whereas in the spleen lymphocytes and systemic antigens enter through the same path and first reach the MZ. It is therefore possible that multiple cells types that pass via this compartment are able to mediate the transfer of intact opsonized antigenic material into the follicles, allowing greater degree of compensation between partially overlapping pathways. Similarly, unlike LNs, where the entry port into the tissue contains only one major subset of macrophages (i.e., expressing CD169), at the spleen site of entry multiple layers of macrophages exit. Therefore, in the absence of SIGN-R1 cells, antigen capture could potentially be compensated by the presence of MARCO macrophages in the marginal zone. The fact that we did not observe any phenotypic defects in pre-GCs and early GCs strongly suggests that antigen delivery to cognate B cells is not obstructed by the absence of SIGN-R1 cells.

5 SIGN-R1 macrophages are required for optimal priming of Tfh responses

5.1 Introduction

T follicular helper (Tfh) cells are crucial for the formation and maintenance of GC responses, as they provide survival signals fundamental to the selection of higher affinity B cells (Mesin et al 2016). There is a strong interdependency between Tfh cells and GC B cells within the GC structure, and therefore defects in either population could result in the collapse of the GC response (Baumjohann et al 2013, Deenick et al 2010, Stebegg et al 2018). Similar to B cells, T cell activation triggers a series of migratory events that guide T cells through specific niches required for optimal Tfh and GC development (Vinuesa et al 2016). Cognate T cells accumulate at the outer T zone as early as 12 hours after activation, where they are initially primed by DCs, and then migrate towards the B/T border at day 2 for a secondary priming event by activated B cells (Li et al 2016). From there T cells migrate towards the interfollicular regions where they proliferate extensively (Coffey et al 2009, Cyster 2010, Vinuesa et al 2016) and finally at around day 5 are found at the follicular centre (Ansel et al 1999) within the GC structure (Vinuesa et al 2016) (Fig.2).

CD4+ DCs preferentially present antigenic peptides to cognate T cells loaded on MHCII and provide co-stimulatory signals, including ICOSL, CD80/86, CD40 and OX40L, which interact with ICOS, CD28, CD40L and OX40 on T cells respectively (Vinuesa et al 2016). Additionally, IL-6 cytokine secretion from antigen presenting cells (APCs) promotes upregulation of BCL6 (Choi et al 2013), the master regulator of Tfh differentiation (Johnston et al 2009, Nurieva et al 2009, Yu et al 2009) as early as

24 to 72 hours after activation (Baumjohann et al 2011, Kitano et al 2011). BCL6 upregulates expression of effector molecules such as PD-1, CXCR5, CXCR4 and SAP (Poholek et al 2010, Yu et al 2009) and represses differentiation into other effector subsets like Th1, Th2 and Th17 (Hatzi et al 2015, Nurieva et al 2009). These early events culminate in maturation of Tfh and their entry into the GC reaction

5.2 Tfh Differentiation in the absence of SIGN-R1 macrophages

Given the crucial role of Tfh cells for an optimal GC response, we considered the possibility that impaired development of GC B cells in SIGN-R1 macrophage-depleted mice may be related to suboptimal priming and differentiation of Tfh cells.

5.2.1 Early activation markers for Tfh development

To determine whether SIGN-R1 macrophages are required for the priming of Tfh cells, we assessed Tfh development after immunisation. We co-transferred macrophage-depleted or sufficient B6 mice with MD4 and OTII splenocytes, followed by HEL-OVA immunisation and analysed splenocytes by flow cytometry 3 days later for PD1+ CXCR5+ Tfh cell frequencies. Although there was a tendency for a reduction in Tfh frequencies in CLL compared to PBS-L-treated mice, these differences were not statistically significant, suggesting Tfh priming is not critically dependent on SIGN-R1 macrophages (Fig.13a). We further examined the phenotype of Tfh cells by comparing the expression of molecules associated with Tfh differentiation. We found no differences in the expression of CD40L, BCL6, CD84 and Ly108 (Fig.13b). In some experiments there was a slight but significant reduction in ICOS expression in the CLL-derived Tfh cells compared to control Tfh cells, however it was not consistent (Fig. 13b). Moreover, this reduction was small, likely not enough to explain our phenotype.

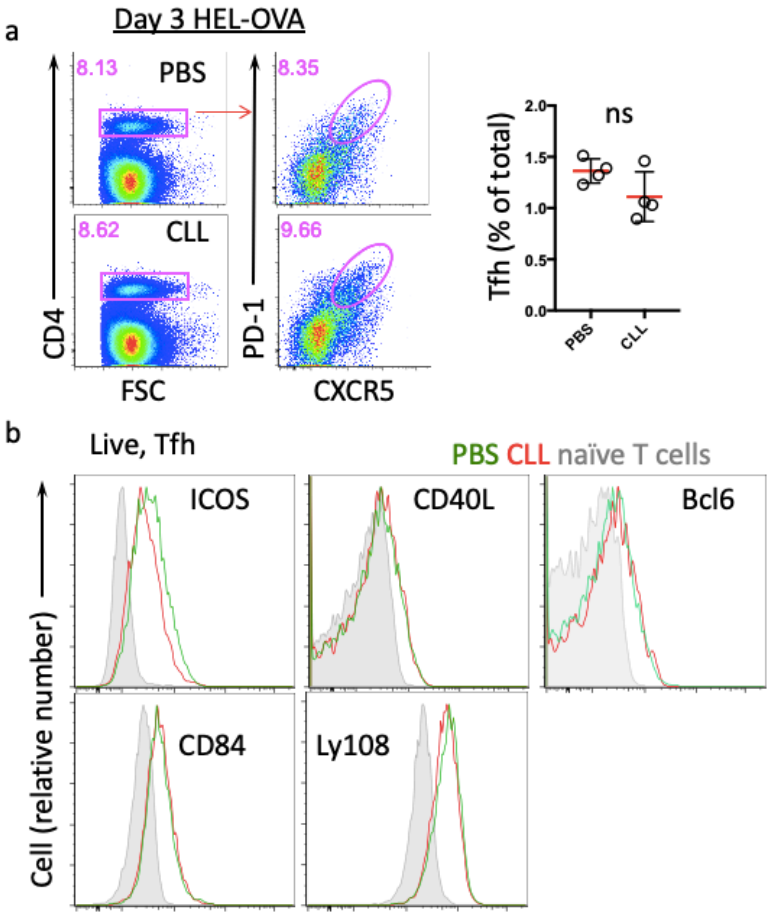


Figure 13. Frequencies and phenotype of Tfh cells in SIGN-R1 macrophage-depleted mice.

B6 mice were treated with PBS-L or CLL 2-4 weeks prior to intravenous co-transfer of MD4 and OTII cells, followed by immunisation with HEL-OVA. Mice were analysed 3 days post immunization. (a) Left, FACS plots showing gating strategy to determine frequencies of Tfh cells. Plots were pre-gated on live cells. Right, percentage of Tfh cells. Data represents one out of 5 experiments performed (b) Histograms, pre-gated on Tfh cells ($PD1^{Hi}CXCR5^{Hi}CD4^{+}$) from 'a' showing expression of ICOS, CD40L, Bcl6 (representative graphs of 5 repeats) and Ly108, CD84 (representative graphs of 2 repeats).

As a more robust approach to explore the impact of SIGN-R1 cell depletion on Tfh development, we applied RNA sequencing. We sorted CD4+CXCR5+PD-1+ Tfh and naïve CD4+CXCR5-PD-1- cells from CLL and PBS-treated mice three days after HEL-OVA immunization (Fig.14). We first focused our analysis on known key

factors in regulating GC B cells. However, our analysis did not reveal difference in the expression profile of these key Tfh differentiation molecules (Fig.14c). There was however a significant ($p \leq 0.01$) fold difference in the expression of 557 genes between Tfh cells from PBS-L and CLL-treated mice. DAVID pathway analysis of these genes revealed enrichment of pathways including “Notch signalling”, “cell cycle”, “B cell receptor signalling”, “glycolytic processes”, “receptor internalisation and signalling”, and “cell-cell adhesion” (Fig.14d).

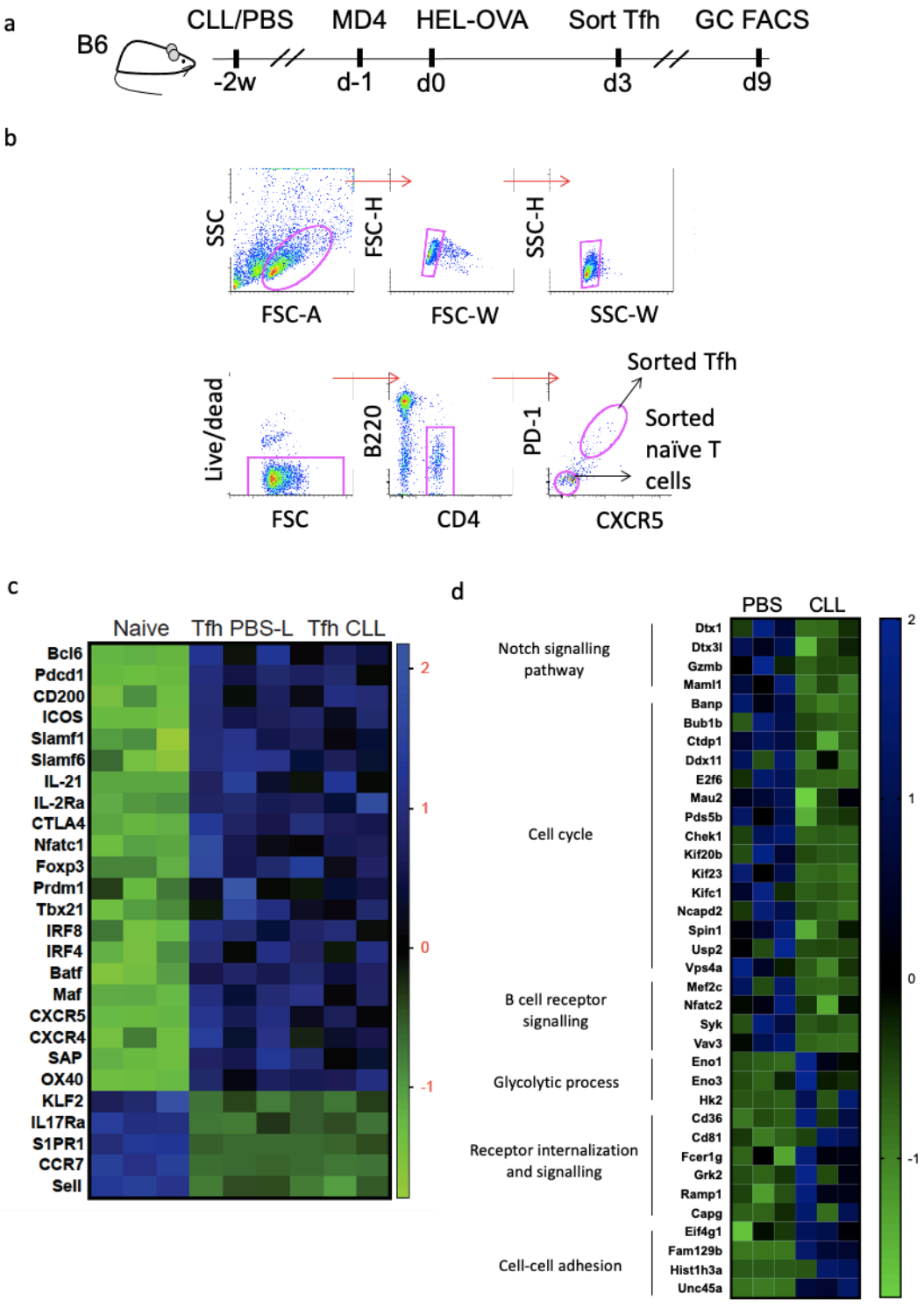


Figure 14. Expression profile and phenotype of Tfh cells in SIGN-R1-depleted mice.

B6 mice were treated with PBS-L or CLL 2 weeks prior to 1x10^6 MD4 cell transfer and HEL-OVA immunization. Spleens were collected on day 3 after immunisation for sort and sequencing (a) Schematic representation of the experiment. (b) Gating strategy for sorting Tfh cells (Live, B220- CD4+ PD-

1^{high} CXCR5^{high}) and naïve T cells (live, B220- CD4++ PD-1^{low} CXCR5^{low}). (c) Heat map of RNA-seq analysis shows expression of Tfh signature genes in naïve T cells compared to Tfh cells from CLL or PBS-L treated mice. Data from each triplicate are shown. (d) Heat map of RNA-seq data of genes that were significantly (p<0.01) up or downregulated in Tfh cells from CLL compared to PBS-treated animals and that were clustered within enriched pathways after David analysis. Data from each triplicate are shown.

5.2.2 Tfh cell localisation

As mentioned in the introduction, T cells undergo a series of migration events required for optimal Tfh differentiation and eventually migrate within the GC structures in the centre of the B cell follicles. To assess whether migration of activated cognate T cells is obstructed in the absence of SIGN-R1 macrophages, we examined the localisation of OTII cells five days after immunisation, the time point preceding the premature GC collapse we observed in SIGN-R1-depleted mice. We enriched CD45.2+ OTII splenocytes for T cells (Fig.15a) and co-transferred them with CD45.1+ MD4 splenocytes into CD45.1+ recipients, followed by HEL-OVA immunisation. As expected, at day 5 after immunisation IgMa+ MD4 cells were positioned in the centre of the follicles, forming early GCs both in PBS-L and CLL-treated animals (Fig.15b). The positioning of CD45.2+ OTII cells was also similar between the two groups – with CD45.2+ cells present within the follicles as well as the IgMa+ germinal centres (Fig.15b). This experiment suggested that the overall migratory behaviour of activated T cells is not affected by the absence of SIGN-R1 macrophages.

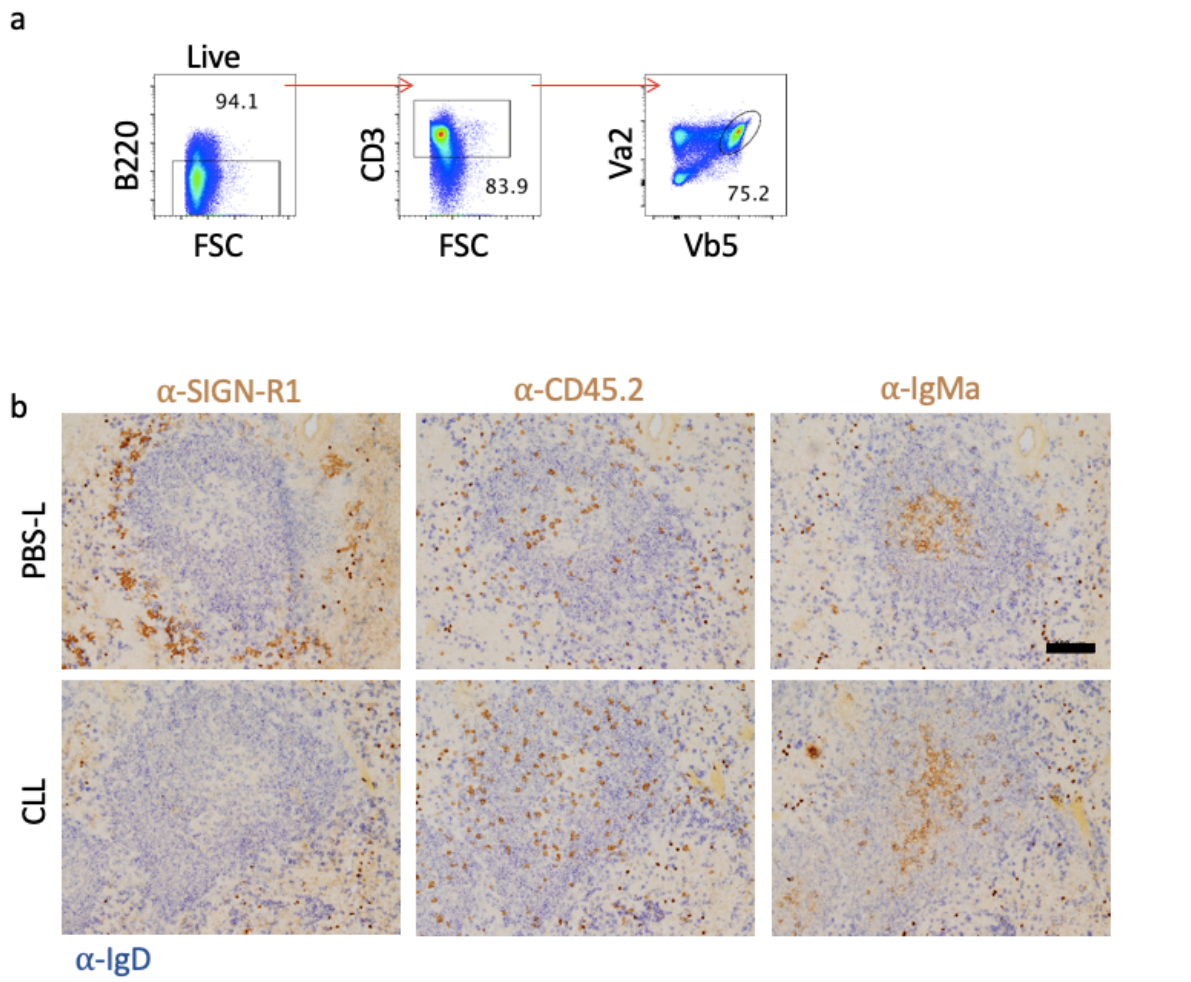


Figure 15. Positioning of Tfh cells in the follicles of SIGN-R1 macrophage-depleted mice

3.6x10^6 CD45.1+ IgMa+ MD4 cells and 3.6x10^6 purified CD45.2+ OTII cells were co-transferred into CD45.1+ recipient mice that were treated with PBS-L or CLL 3 weeks earlier. The next day, mice were immunized with HEL-OVA. (a) Enrichment efficiency of OTII splenocytes before transfer, graphs are pregated on live cells. (b) Five days post immunization, spleens were frozen and consecutive sections were stained for IgD (blue) and IgMa (to identify transferred MD4 B cells, brown), CD45.2 (to identify transferred OTII cells, brown) or SIGN-R1 (brown). Scale 100µm. Shown one representative example out of 2 experiments performed. Sections from spleens of at least 3 mice were analysed in each experiment.

5.2.3 T follicular regulatory cell development

Importantly, Tfh cells also comprise a subpopulation with suppressive capacities, known as T follicular regulatory cells (Tfr). Studies suggest that Tfr cells derive from natural regulatory T cells and not from naïve or Tfh cell populations and are important for suppressing the GC response against self-antigens (Linterman et al 2011, Wollenberg et al 2011). Tfr cells can supress Tfh numbers and increase the proportion of antigen-specific GC B cells (Linterman et al 2011) as well as decrease the magnitude of the GC response (Ding et al 2014). Therefore, it was important to test whether the ratio of Tfr to Tfh cells was skewed in the absence of SIGN-R1 macrophages. We analysed the relative frequency of Tfr cells (defined as CD4+FOXP3+PD-1+ CXCR5+) in macrophage-depleted or sufficient mice 5 days after SRBC immunisation. The frequencies of Tfr cells were similar between the two groups (Fig.16) suggesting that Tfr differentiation is not affected by the absence of SIGN-R1 cells.

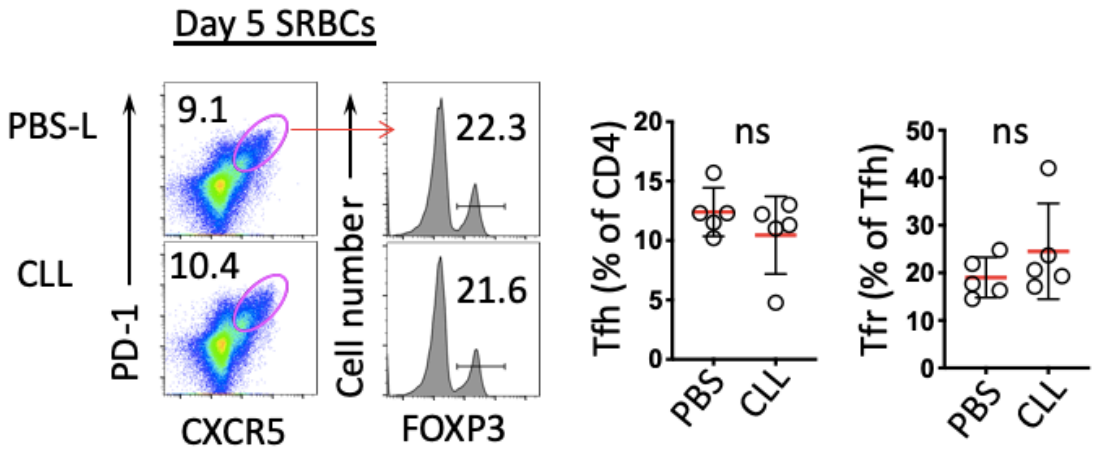


Figure 16. Frequencies of Tfh and Tfr in SIGN-R1 macrophage-depleted mice

B6 mice were treated with PBS-L- loaded liposomes or CLL 3-4 weeks prior to immunization with SRBCs. Mice were analysed 5 days post immunization. Left, FACS plots showing gating strategy to determine frequencies of Tfh and Tfr cells, pre-gated on CD4+ live cells. Right, percentage of Tfh and Tfr cells. Data represents 1 out of 3 experiments performed.

5.3 Specific pre-priming improves the GC response in macrophage-depleted mice

While the above experiments did not reveal any obvious defects in Tfh development in the absence of SIGN-R1 macrophages, we considered the possibility that subtle defects may not be easily detected using the above approaches. Therefore, to indirectly test whether optimal Tfh cells responses may require SIGN-R1 macrophages we explored whether induction of Tfh cells prior to macrophage depletion will rescue the GC B cell response. To this end we generated OVA-specific Tfh cells prior to macrophage depletion by pre-priming mice with Ova antigen 2 weeks before macrophage depletion. After 2 weeks, when the splenic architecture begins to recover, but SIGN-R1 macrophages are still missing, we transferred MD4 cells and immunised with HEL-OVA antigen. We analysed GCs by flow cytometry and histology 8 days later. We observed significant increase in GC frequencies when mice were pre-primed with OVA prior to CLL injection compared to CLL-treated mice that have not been pre-primed (Fig.17a-c). By histology, GL7+ GCs structures were also abundant in the pre-primed CLL-treated animals (Fig.17d). This data shows that specific pre-priming can ameliorate the GC collapse phenotype.

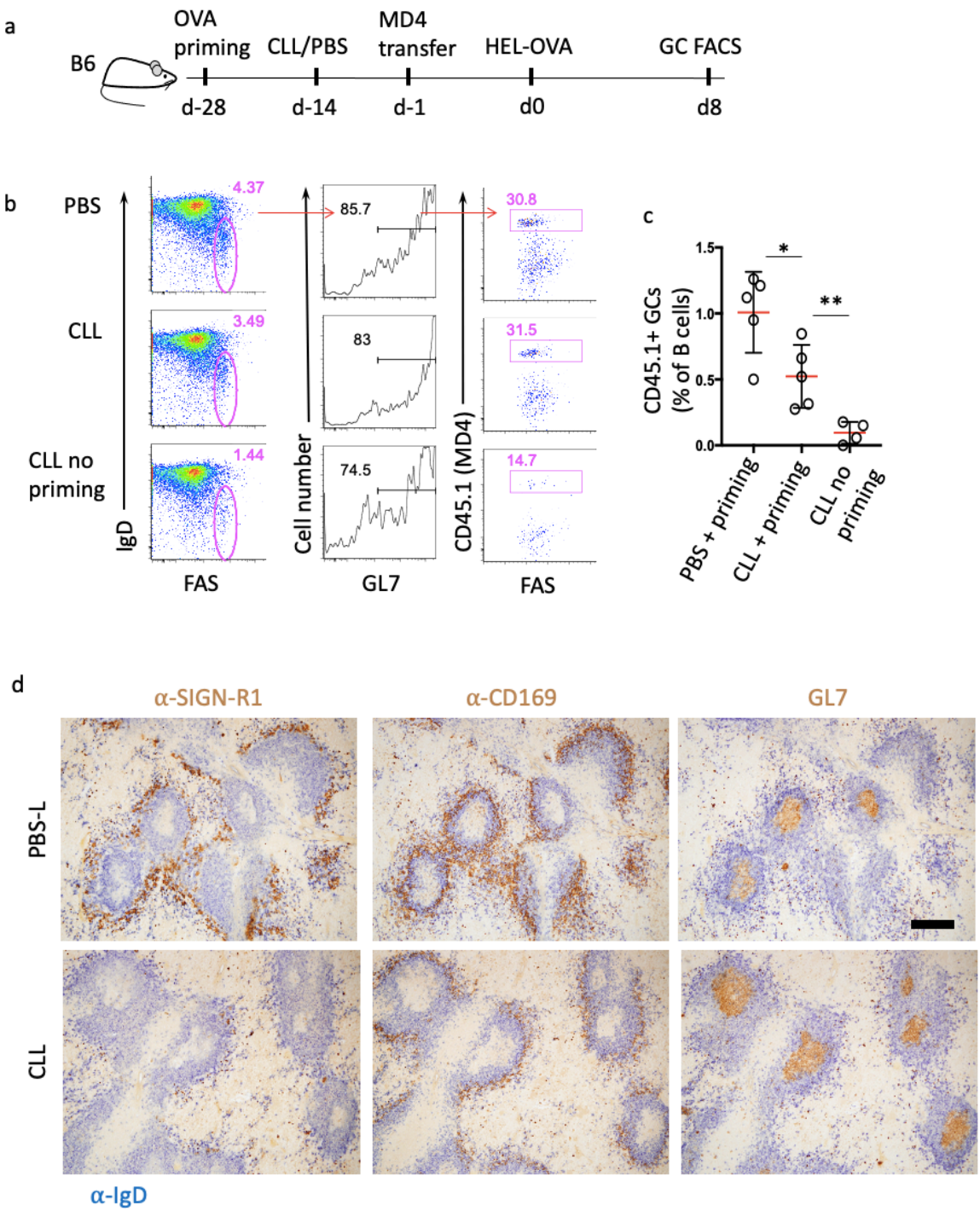


Figure 17. Specific pre-priming rescues the GC response in the absence of SIGN-R1 macrophages

B6 mice were treated with clodronate or PBS-loaded liposomes 2 weeks prior to priming with OVA in Ribi adjuvant. 2 weeks later, both groups were adoptively transferred with 1x10^6 MD4 cells and immunised with HEL-OVA in Ribi adjuvant. Spleens were collected and analysed by FACS and histology 8 days later. (a) Schematic representation of the experiment. (b) FACS plots showing gating strategy to determine

the frequencies of CD45.1+GL7+FAS+IgD^{Low} GCs. (c) Percentage of CD45.1+ GCs of B220+ B cells. (d) Consecutive spleen sections stained for IgD (blue), SIGNR1 (brown), CD169 (brown) and GL7 (brown). Scale bar 200um. Shown 1 representative example of 3 experiments performed. Sections from spleens of at least 3 mice were analysed in each experiment.

This rescue effect could be due to induction of specific Tfh help induced prior to macrophage depletion or, due to the induction of non-specific mechanisms such as the formation of pre-existing GC structures that can enhance subsequent responses. To distinguish between these possibilities, we next tested the effect of non-specific pre-priming. We primed mice with SRBC, which generates SRBC-specific Tfh cells as well as large pre-existing GC structures, followed by macrophage depletion and immunisation with PE antigen (Fig.18). Under these conditions, the PE-specific GC response in CLL-treated mice were not recovered. This suggests that the partial rescue of the GC B cell development we observed in the OVA pre-priming system depends on the formation of antigen-specific Tfh cells.

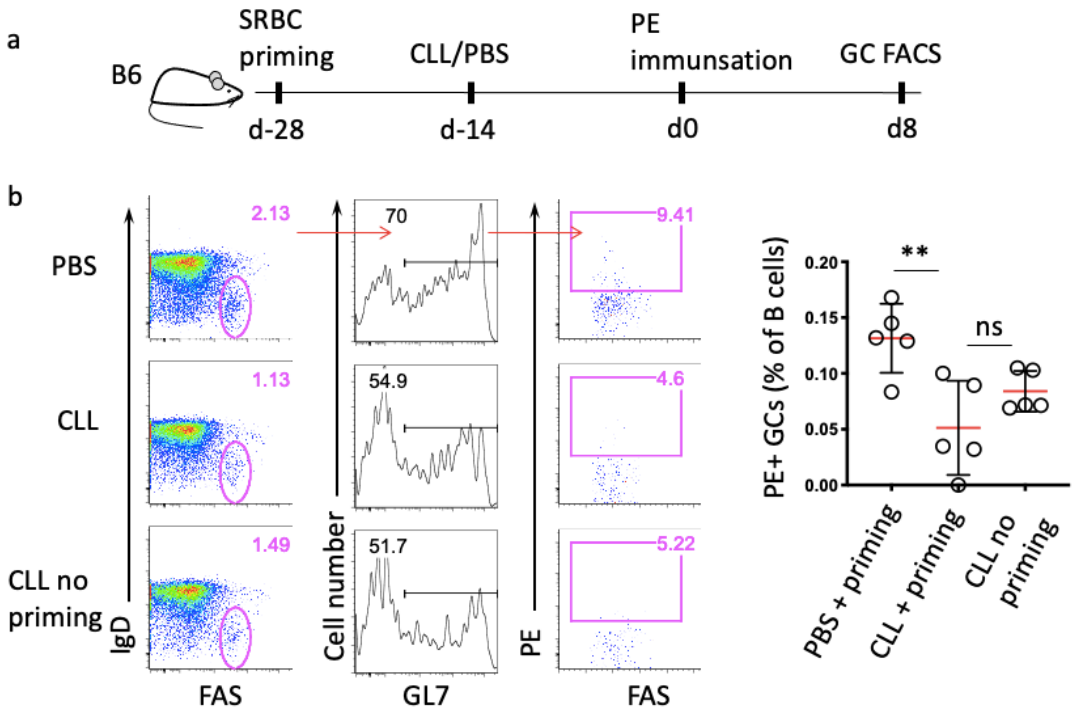


Figure 18. Non-specific pre-priming cannot rescue the GC response in the absence of SIGN-R1 macrophages

Mice were pre-primed with SRBCs 2 weeks prior to CLL or PBS-L treatment. Two weeks later, mice were immunized with PE and analysed by flow cytometry 8 days later. (a) Schematic representation of the experiment. (b) Left, gating strategy to determine the frequencies of PE-specific GC B cells. Right, quantification of frequencies of PE^+ GC B cells. Shown is one representative experiment out of 3 performed.

5.4 Enhancing APC-Tfh cell interactions boosts the GC response in SIGN-R1-depleted mice

To further test the above hypothesis, we used complementary approaches to ask whether boosting the Tfh response will help to overcome the loss of SIGN-R1 cells and restore GC B cell responses. Tfh cells require persistent TCR stimulation by APCs for maintenance and survival (Baumjohann et al 2013, Deenick et al 2010). To test whether enhancing antigen presentation could improve the GC response in the absence of macrophages we delivered the OVA antigen targeted to APCs by conjugating it to an antibody against the receptor DEC205, which is expressed on CD8+ DCs and GC B cells. This treatment has been previously shown to strongly enhance Tfh responses (Bonifaz et al 2004, Victora et al 2010). We co-transferred MD4 and OTII splenocytes into PBS-L or CLL-treated mice, followed by HEL-OVA immunisation (Fig.19). On day 4, the timepoint before GC collapse, we injected the OTII specific protein conjugated to DEC205 antibody (aDEC205-OVA) and analysed GC B cells frequency 4 days later. There was a significant increase in GC B cells frequencies in aDEC205-OVA-treated mice compared to CLL-treated mice that did not receive the boost injection (Fig.19). This data adds support to the possibility that priming of Tfh cells is suboptimal in the absence of SIGN-R1 macrophages.

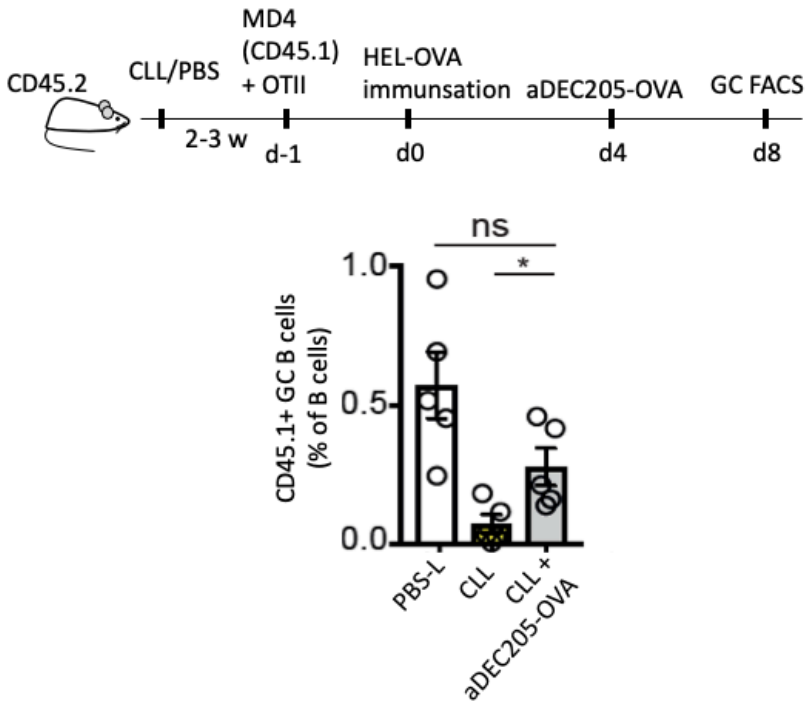


Figure 19. Enhancing Tfh-APC cell interactions boosts the GC response in SIGN-R1-depleted mice

Hosts (CD45.2$^+$) were treated with CLL or PBS-L. After 2-3 weeks, mice were transferred with CD45.1$^+$ MD4 B cells and immunized with HEL-OVA. Four days later, mice were i.v injected with or without chimeric anti-DEC205 antibody fused to an OVA-derived peptide. GC frequencies were analysed on day 8 after immunisation. Top, schematic representation of the experiment. Bottom, frequencies of CD45.1$^+$ GC B cells. Figure shows one representative experiment out of 3 performed.

5.5 Activated CD4+ T cell transfer improves the germinal centre response in macrophage-depleted mice

If the above hypothesis is correct, we expect that transfer of antigen-specific Tfh cells from macrophage-sufficient mice could save the GC response in macrophage-depleted spleens. To test this, we immunised donor B6 mice with OVA antigen to generate OVA-specific Tfh cells and 2 weeks later collected the spleens and enriched them for CD4+ T cells. We then transferred these cells together with MD4 splenocytes into

macrophage-sufficient or deficient mice, followed by HEL-OVA immunisation (Fig.20). The frequencies of GC B cells in SIGN-R1-depleted mice were significantly increased in mice that received CD4+ T cells compared to those that were transferred with MD4 cells alone. Furthermore, the CD4-mediated GC boost was only observed in macrophage-depleted mice as activated CD4 transfer had no effect on the strength of the response in macrophage-sufficient mice (Fig.20). This data further supports the notion that SIGN-R1 macrophages are important for optimal Tfh cell development.

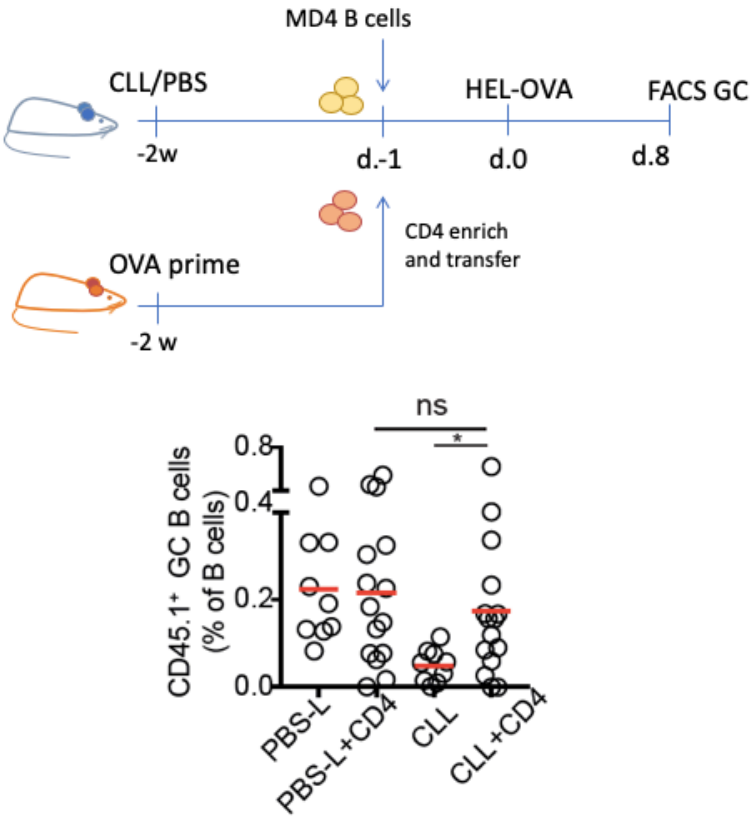


Figure 20. Activated CD4+ T cell transfer improves the GC response in SIGN-R1-depleted mice

Mice (CD45.2$^+$) treated with CLL or PBS-L 3 weeks earlier were transferred with CD45.1$^+$ MD4 B cells with or without co-transfer of CD4 T cells (CD45.2$^+$) that were enriched from mice immunized with OVA 2 weeks earlier. Recipients were immunized with HEL-OVA and spleens were analysed 8 days later to determine the frequencies of CD45.1$^+$ GC B cells. Top, schematic representation of the experiment. Bottom, Frequencies of CD45.1+ GC B cells. Data is pooled from 3 independent experiments.

5.6 Conclusions and discussion

In this chapter we explored the possibility that SIGN-R1 macrophages are required for optimal Tfh cell development and thus regulate the GC response. We found no major defects in Tfh differentiation in the absence of SIGN-R1 cells, as shown by similar Tfh frequencies at day 3 and day 5 post immunisation as well as similar protein and mRNA expression levels of known Tfh activation and differentiation factors at day 3 after immunisation. Antigen-specific T cells in CLL-treated mice were localised to the B cell follicles at day 5 after immunisation, further suggesting Tfh development was normal. However, we observed that the GC collapse phenotype in SIGN-R1-depleted animals was partially ameliorated by priming mice before macrophage depletion and immunisation. This effect was antigen-specific as priming with an unrelated antigen had no effect on the immunisation, which suggests that Tfh development after CLL treatment was defective. In support of this hypothesis, boosting Tfh-APC interactions by injecting aDEC205-OVA or delivering exogenous Tfh cells also improved the GC response. Altogether this data suggests that optimal Tfh development requires SIGN-R1 cells.

While this data strongly suggests of a suboptimal Tfh response, we did not identify the specific molecular mechanism that underlines the defects in Tfh responses when SIGN-R1 cells are missing. Addressing this question is complicated by the dynamic nature of the GC process and the limitations associated with analysis that focuses on a restricted time point - our data was limited to snapshots of the Tfh differentiation pathway and known activation markers and therefore may have missed subtle changes that are best captured at more specific critical time points. For example, the Tfh sequencing data at day 3 post immunisation might precede the potential developmental defect. We selected

this timepoint because we were concerned that a later timepoint might reveal Tfh differentiation defects which are a consequence rather than the cause of GC collapse. Highlighting the dynamic nature of the GC response and the importance of delivering signals at a specific timepoint during differentiation, a recent study showed that CD4+ T cells with identical TCRs can be preferentially polarised into Tfh or Th1 cell type depending on whether they were exposed to type I interferon withing the first 24 hours of activation, leading to preferential humoral or cellular adaptive immune response in different viral infections (De Giovanni et al 2020). Furthermore, while our Tfh sequencing data did not reveal any obvious candidate genes, there were significant differences in the expression of over 500 genes between Tfh from CLL-treated and control mice, that currently have no established direct link with the Tfh differentiation program. Future studies could analyse these genes and potentially identify new factors involved in Tfh maintenance. The fact that we used bulk Tfh cells, rather than scRNA analysis, might also have limited our ability to define more subtle changes that occur in specific subsets of cells within this population. Furthermore, a sequencing approach might not be able to reveal potential differences in protein levels as more than 50% of protein expression in activated T cells is estimated to occur as a result of post-transcriptional regulation of mRNA stability and translation rather than transcription (Cheadle et al 2005).

Importantly, in the activated CD4 transfer experiments, T cell transfer boosted the CLL but not the PBS group, which suggests that this help relates to the SIGN-R1 depletion phenotype, rather than a general boost of the immune system. Altogether this data provides strong evidence that Tfh cells are sub-optimally induced in the absence of SIGN-R1 macrophages.

6 SIGN-R1 cells regulate the positioning of 33D1+ dendritic cells in the spleen

6.1 Introduction

As discussed in the introduction (Chapter 1.5.3), cDC2 cells are the primary antigen presenting cells important for the activation of CD4+ T cell responses, including initiating Tfh differentiation. After sensing inflammatory mediators such as inflammatory cytokines, pathogens or tissue damage, resting DCs mature by upregulating surface expression of MHCII, costimulatory molecules CD86 and CD40 which makes them competent of activating cognate CD4+ T cells (Banchereau & Steinman 1998, Sporri & Reis e Sousa 2005). CD83 is also upregulated on DCs and has a role in stabilising MHCII and CD86 surface expression (Tze et al 2011).

cDC2 localisation at steady state is essential for their function and maintenance and is mediated by expression of EBI2 which guides cells towards its ligands 7α,25-OHC and 7α,27-OHC (Gatto et al 2013, Lu et al 2017, Yi & Cyster 2013). cDC2 cells in EBI2 receptor-deficient and EBI2 ligand-deficient mice position predominantly in the T zone and red pulp of the spleen instead of the interfollicular regions (Gatto et al 2013, Yi & Cyster 2013). This mislocalisation leads to decreased DC survival as B cell-derived lymphotoxin (LT) signalling within the interfollicular regions is important for cDC2 maintenance (Kabashima et al 2005). Failure to position at the interfollicular regions also results in inefficient antigen capture and CD4+ T cell priming (Gatto et al 2013, Yi & Cyster 2013).

After activation cDC2 rapidly upregulate the chemokine receptor CCR7, which directs them towards the T zone compartment of the white pulp. DCs that lack CCR7 fail to induce T cell proliferation in vivo, highlighting the importance of this migration for T-dependent immune responses (Calabro et al 2016b). EBI2 upregulation on activated cDC2 cells further navigates their positioning towards the outer regions of the T zone, while CD8+ DCs in the T cell zone centre restrict cDC2 entry to the inner T zone by producing HSD3B7, the enzyme that metabolises and inactivates EBI2 ligands (Lu et al 2017).

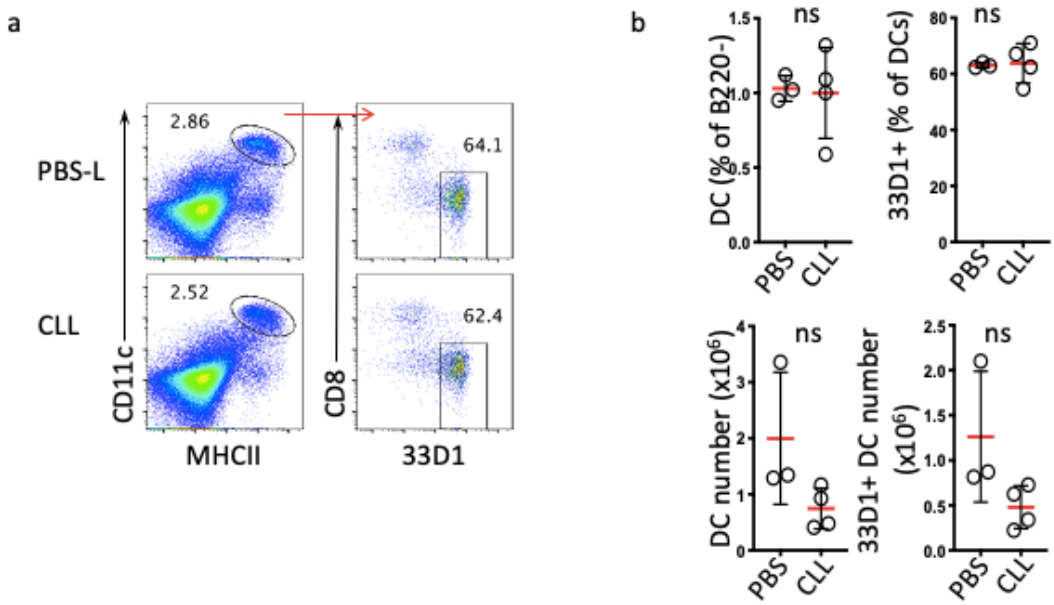
In addition to directly activating CD4+ T cells, mature cDC2 cells promote Tfh differentiation by reducing the available IL-2 cytokine secreted by activated T cells (Li et al 2016). IL-2 is a potent inhibitor of Tfh differentiation and promotes differentiation into other effector phenotypes (Ballesteros-Tato et al 2012, Liao et al 2013). By producing membrane-bound and secreted forms of CD25, the receptor for IL-2, activated cDC2 cells reduce IL-2 concentrations locally, in the environment of newly activated CD4 T cells, promoting Tfh differentiation and thus GC responses (Li et al 2016)

6.2 33D1+ DC cell localization is altered in the absence of SIGN-R1 cells

Given that cDC2 cells are essential for CD4+ T cell priming and Tfh differentiation, and that they reside in close proximity to SIGN-R1 macrophages at steady state, we hypothesised that cDC2 functions may be regulated by SIGN-R1 macrophages, and that loss of this regulation when SIGN-R1 cells are absent, may contribute to suboptimal Tfh priming and GC responses.

6.2.1 CLL macrophage ablation

First, we assessed whether SIGN-R1 macrophages are required for the maintenance and positioning of cDC2 cells at steady state. We depleted SIGN-R1 macrophages using clodronate liposomes and assessed DC frequencies and localisation 3 weeks later. We found that the frequencies and numbers of total DCs (MHCII+CD11c+) as well as 33D1+ cDC2 cells were similar between CLL and PBS-L-treated controls (Fig.21a,b). Importantly however, the positioning of 33D1+ cells within the spleen was drastically affected by SIGN-R1 depletion, with DCs found predominantly within the MZ rather than at the interfollicular regions (Fig.21c). This data suggests that SIGN-R1 cells are important for the correct localisation of Tfh-priming DCs.



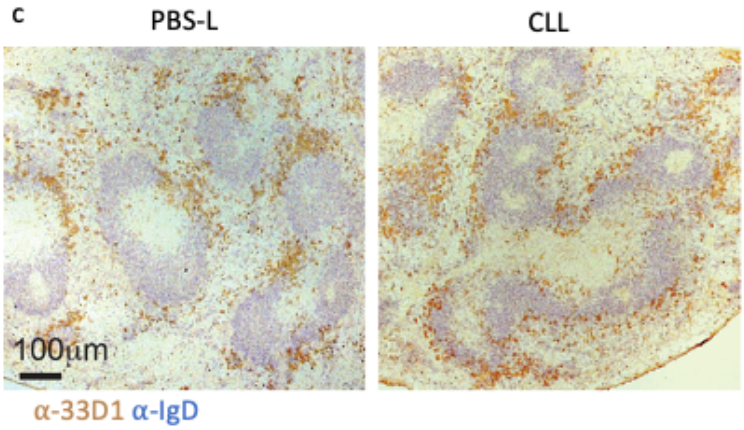


Figure 21. DC positioning is altered after CLL treatment

B6 mice were treated with clodronate or PBS-loaded liposomes and 3 weeks later spleens were harvested. (a) FACS plots showing gating strategy to determine the frequencies of MHCII+CD11c+ DCs and 33D1+ DCs. Pre-gated on live, B220- cells. (b) Frequencies and numbers of total and 33D1+ DCs. (c) Spleen sections stained for IgD (blue) and 33D1 (brown). Scale bar 100um. Shown 1 representative experiments of 3 independent repeats, 3-5 mice per group.

6.2.2 SIGNR1-DTR macrophage ablation

We next wanted to confirm the above data by specifically depleting SIGN-R1 cells using the SIGN-R1-Cre/DTR system. We treated SIGN-R1-Cre/DTR mice with DT 10, 5 and 1 day before collecting the spleens in order to give time for any cell death-induced inflammation to subside (Chatziandreou et al 2017) and compared the frequencies and positioning of 33D1+ DCs with untreated genotype and sex-matched controls. Again, we found that, although the numbers or frequencies of 33D1+ cells were not affected by the treatment (Fig.22a), their positioning was skewed towards the MZ region (Fig.22b), further supporting the possibility that SIGN-R1 expressing cells are necessary for their regulation. Of note, while the general phenotype was similar between the two depletion models, in the SIGN-R1-DTR depletion system, 33D1+ DCs were also found within the T zone (Fig.22b), possibly due to the acute nature of the ablation in this system, which leads to local inflammation and potentially partial activation of DCs.

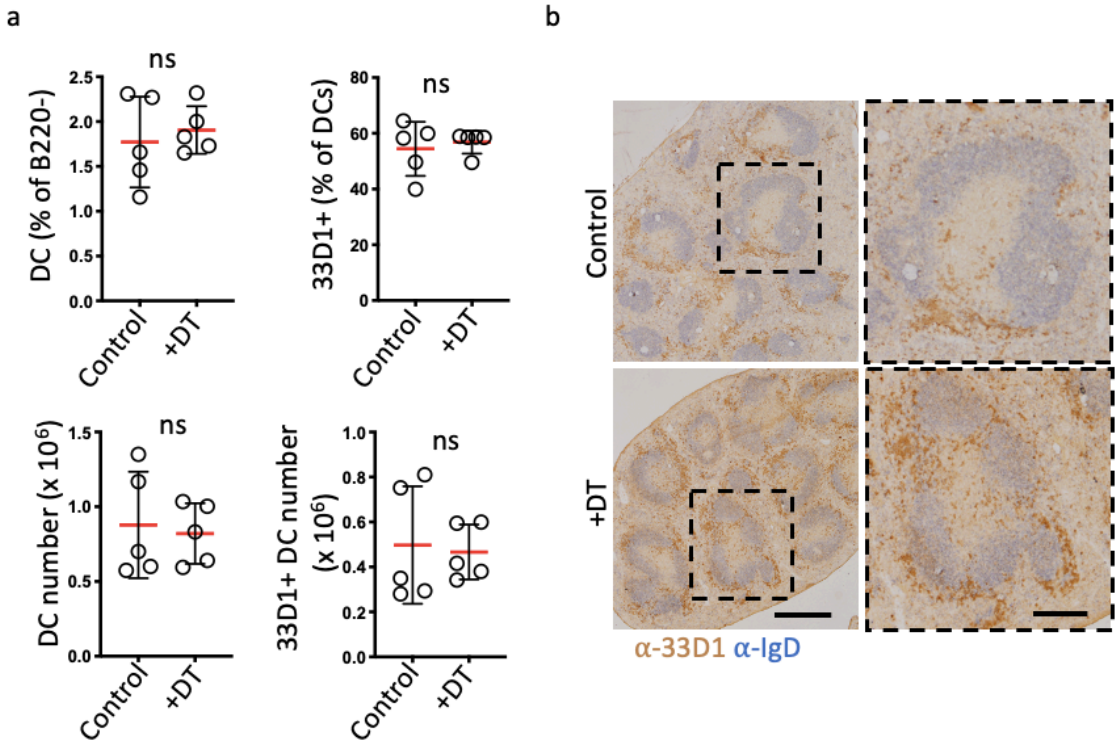


Figure 22. DC positioning is altered after specific SIGN-R1 depletion.

SIGN-R1-Cre/DTR mice were treated or not with DT 10, 5 and 1 day before spleens were harvested. (a) Frequencies of cDCs (defined as B220- CD11c+ MHCII+) and cDC2 (defined 33D1+ cDCs). (b) Spleen sections stained for IgD (blue) and 33D1 (brown). Right images show higher magnification. Scale bar 500um (left), 200um (right). Shown 1 representative experiment of 3 independent repeats, 3-5 mice per group.

6.3 Dendritic cell phenotype and localization after immunisation

6.3.1 DC antigen acquisition and activation

To reveal potential functional defects in 33D1+ DCs in the absence of SIGN-R1 cells, we assessed the capture of antigen from the blood and DC activation by flow cytometry.

We injected intravenously SRBC fluorescently labelled with PKH26 dye into CLL or PBS-L-treated mice and analysed DC activation 3 hours later. 33D1+ DCs from PBS and CLL mice were labelled to a similar extent with PKH26 (Fig.23a), suggesting antigen acquisition is not severely altered in SIGN-R1-depleted animals. Furthermore, we found no significant difference in the activation status of PKH26-labelled 33D1+ DCs as shown by the similar level of upregulation of MHCII, CD80, CD86 and CD83, as well as by the upregulation of the chemokine receptor CCR7 (Fig.23a), which suggests that DCs are maturing and becoming capable of migrating towards the T zone.

6.3.2 DC positioning after immunisation

As discussed in the introduction, CCR7 receptor upregulation is one of several factors that work together to facilitate optimal positioning of 33D1+ DCs, including EBI2 upregulation and maintenance of EBI2 ligand gradient at the border of the T zone. To definitively test whether DC migration is intact in the absence of SIGN-R1 cells, we assessed 33D1+ DC localisation at day 1 after SRBC immunisation, a timepoint at which they are localised at the outer T zone (Li et al 2016). We found that 33D1+ cells localised similarly to PBS-L-treated controls, at the periphery of the T zone (Fig.23b). Thus, despite being displaced from their natural niche in the interfollicular regions in the absence of SIGN-R1 cells, under these conditions 33D1+ DCs appear to be able to capture and process antigenic material.

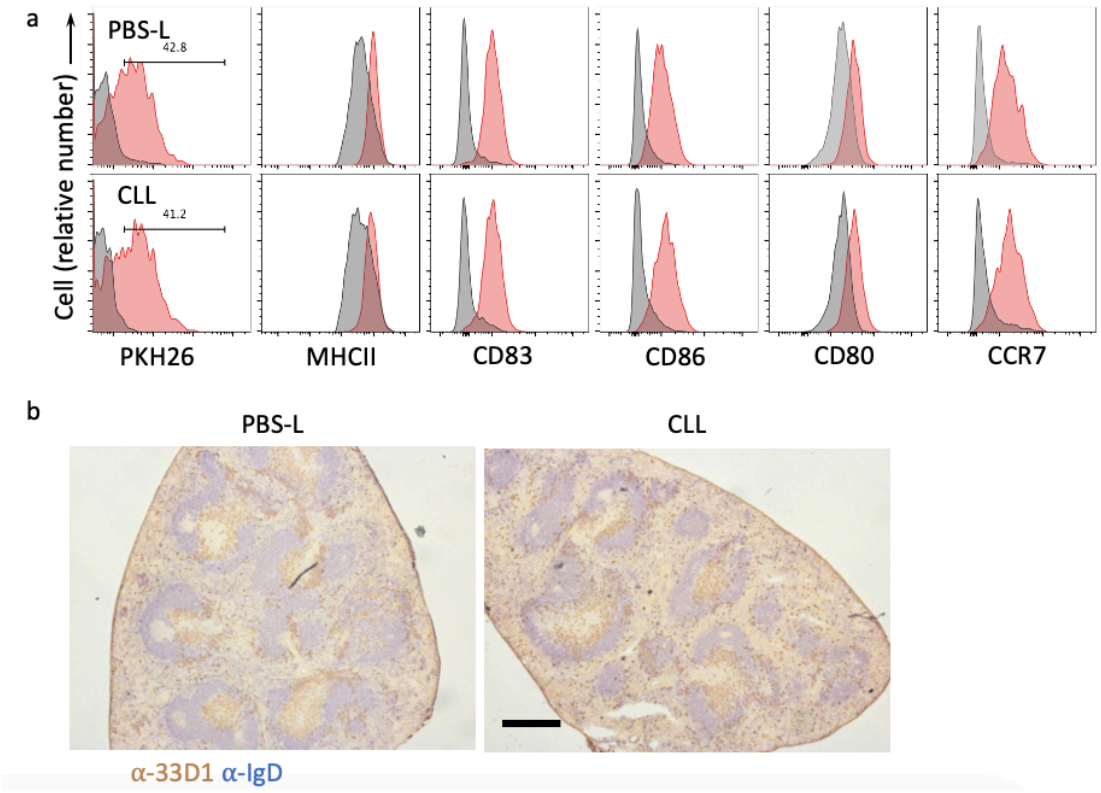


Figure 23. Antigen acquisition, activation and positioning of 33D1+ DCs after immunisation.

(a) Mice that have been treated with PBS-L (top) or CLL (bottom) 2-3 weeks earlier as above were immunized with PKH26-labeled SRBCs. Three hours later spleens were harvested and analysed by flow cytometry. Histograms pregated on live, B220-CD11c+MHCII+33D1+ cells. PKH26-SRBC-transferred spleens (red), no transfer PBS control (gray). Shown one representative example out of 5 independent experiments performed. (b) B6 mice were treated with CLL or PBS-L 2 weeks prior to immunisation with SRBC. Spleens were collected 24 hours later and stained for IgD (blue) and 33D1 (brown). Scale 500um. Shown 1 representative experiment of 2 independent repeats, 3-4 mice per group.

6.4 Recovery of SIGN-R1 macrophages restores DC positioning and numbers and saves the GC response.

Localization of 33D1+ DCs to the interfollicular regions in the spleen is necessary for their survival (Kabashima et al 2005). However, displacement of the cells from these

sites using CLL or DT mediated ablation of SING-R1 cells did not lead to reduced numbers or frequencies of 33D1+ DCs (Fig.21a, 22a). We hypothesized that this may be due to the relatively limited time in which the cells were displaced under these conditions (1-3 weeks). To test this possibility and to further explore the role of SIGN-R1 macrophages in regulating GC B cell responses by promoting 33D1+ DC positioning at the interfollicular sites, we looked for an approach that would allow us to selectively deplete SIGN-R1 macrophages for longer periods of time and that would permit us to re-populate the compartment with SIGN-R1 macrophages in a controlled manner. As mentioned in the introduction, previous studies have shown that homeostatic maintenance of SIGN-R1 macrophages in the spleen depends on the presence of MZB cells and that in CD19 KO mice, in which MZ B cells fail to develop, SIGN-R1 macrophages (but not CD169+ or SIGN-R1-MARCO+ cells) are selectively diminished (Martin & Kearney 2000, You et al 2011, You et al 2009). Furthermore, while the gross follicular architecture in this mouse model is normal, CD11c+ cells in CD19 KO mice are aberrantly distributed in the MZ instead of the interfollicular regions (You et al 2009), similar to the positioning we observed in the CLL and SIGN-R1-DTR depletion systems. Importantly, these defects could be restored when the MZB cell niche was reconstituted by adoptive transfer of WT B cells (Arnon et al 2013, You et al 2009). As such, CD19 KO mice represent a useful model allowing us to test the consequences of long-term SIGN-R1 macrophage depletion on the GC response and DC positioning as well as to explore how SIGN-R1 recovery may affect these processes.

6.4.1 SIGN-R1 subset reconstitution in CD19 KO mice

Although previous studies already showed that CD19KO mice have reduced SIGN-R1 macrophages and that this can be rescued by reconstitution of the MZB cell

compartment, a quantitative assessment of these effects has not been reported and the extent of the defect remains unclear. We therefore wanted to validate these reported results and to obtain a more quantitative estimation for the dependency between MZB cells and SIGN-R1 macrophage survival by comparing their frequencies before and after MZB cell reconstitution of CD19KO mice. As previously reported, 2 weeks after transfer of WT splenocytes into CD19 KO mice, a sizable population of MZB cells developed (Fig.24a). To determine the effect on SIGN-R1 cell loss, we performed quantitative immune fluorescence analysis, where we calculated the density of SIGN-R1 cells before and after MZB cell reconstitution (Fig24b). It was essential to use histological based analysis because flow cytometry assessment of SIGN-R1+ cells in the spleen proved highly inconsistent. This analysis revealed that when MZB cells were absent (CD19KO), SIGN-R1 macrophage frequencies drop by more than 50%, a defect that was effectively corrected following MZB cell reconstitution.

6.4.2 Aberrant DC positioning in CD19 KO mice is restored after SIGN-R1 reconstitution

Previous studies have demonstrated that the total CD11c+ cells within the spleens of CD19 KO mice are mispositioned (You et al 2009), however a specific defect in 33D1+ DCs has not been shown. By assessing 33D1+ DC positioning in CD19 KO mice using histology we observed a clear 33D1+ cell positioning predominantly in the marginal zone (Fig.25a). Furthermore, reconstitution of the MZ restored 33D1+ positioning at the bridging channels (Fig.25a). This data is consistent with our hypothesis that SIGN-R1 macrophages regulate 33D1+ DC positioning.

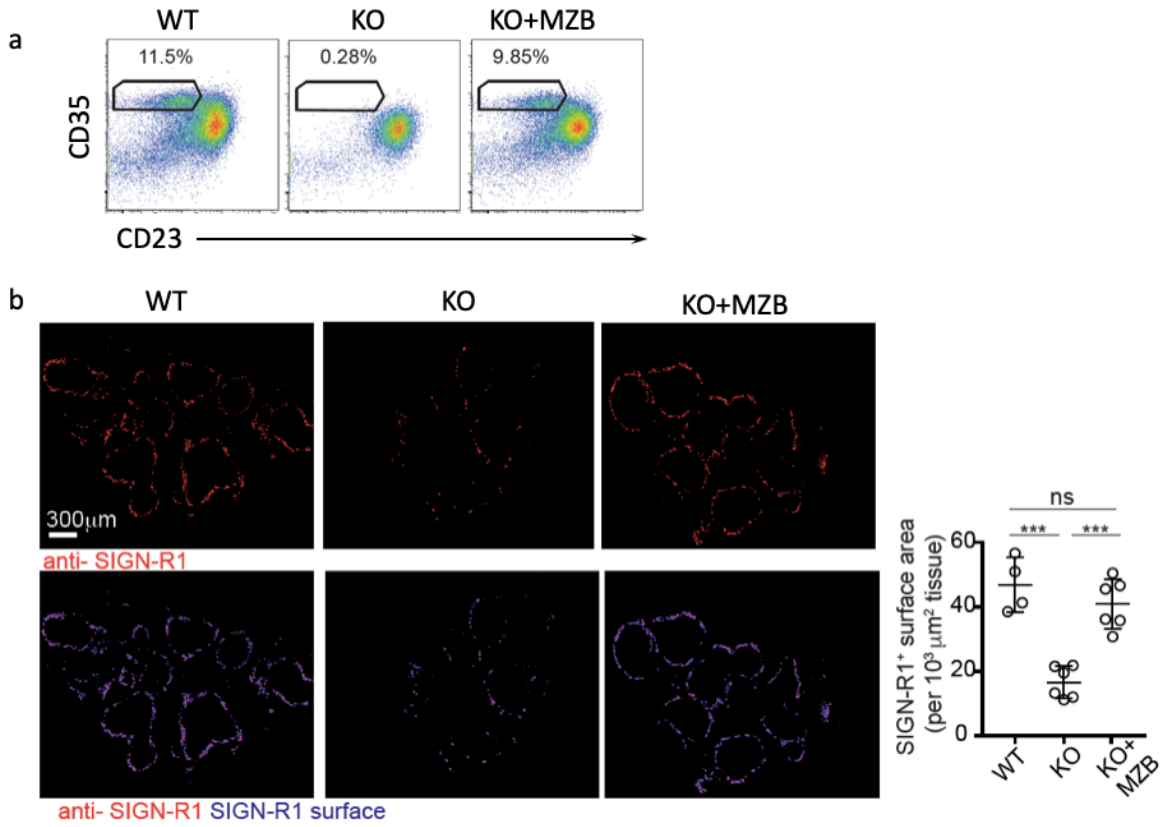


Figure 24. MZB reconstitution in CD19 KO mice restores SIGN-R1 macrophages

Spleen sections from WT, CD19 KO and CD19 KO mice that have been reconstituted with WT splenocytes to restore MZB cells (+MZB) (a) Flow cytometry gating on MZB cells, gated as $CD23^{Low}$ $CD35^{Hi}$, pregated on live, B220+ cells. (b) WT, CD19 KO and MZB reconstituted mice were stained with anti-SIGNR1 (red, top panel). Surfaces surrounding SIGN-R1 positive cells (SIGN-R1-surface, blue) were created using the Imaris software. On the right, quantification of the frequencies of SIGN-R1+ cells, calculated as the sum of SIGN-R1-surface areas (um^2) normalized to the total surface area of the section (um^2). Each circle represents data from one analysed section.

6.4.3 MZ reconstitution restores 33D1+ DC numbers in CD19 KO mice

The positioning of 33D1+ DCs within the MZ was more pronounced in CD19 KO mice compared to our SIGN-R1 depletion systems. This could be explained by the fact that CD19 KO mice have never had a functional MZ, and only a few SIGN-R1 macrophages, and therefore DC positioning has been defective for a longer period

compared to the CLL and DTR depletion systems. In support of this hypothesis, we observed that the numbers of 33D1+ DCs in CD19 KO mice were significantly reduced compared to WT animals, and were restored after reconstitution (Fig.25b). This data is in agreement with previous findings that positioning of 33D1+ DCs at the interfollicular regions facilitates the delivery of survival signals from follicular B cells (Gatto et al 2013, Yi & Cyster 2013) and suggests that SIGN-R1 cells indirectly support DC maintenance.

6.4.4 MZ reconstitution restores the GC response in CD19 KO mice

To examine how SIGN-R1 reconstitution and recovery of DC positioning in CD19 KO mice affects the GC response, we compared GC frequencies in CD19 KO mice before and after reconstitution with WT B cells. Because the endogenous B cells in CD19KO mice are significantly intrinsically impaired in their BCR signalling and their ability to form germinal centres (Depoil et al 2008, Rickert et al 1995) we co-transferred MD4 and OTII cells and assessed the GC response of the transferred WT MD4 B cells after HEL-OVA immunisation. At day 8 post immunization, we analysed the frequencies of GC B cells and found that GC formation was significantly impaired in CD19KO mice, with almost undetectable GC frequencies, while the reconstituted group had a clear GC population of ~3% (Fig.25c). This data is consistent with our observations that SIGN-R1 macrophages are necessary for GC responses and provide further support to the hypothesis that their ability to regulate cDC2 positioning and function is important for optimal Tfh differentiation and GC development.

6.5 DC-independent germinal centre responses do not require SIGN-R1 cells

To further test this model, we used an immunisation system in which DCs are dispensable for Tfh priming (Hong et al 2018). Until recently DC were considered the main APC subset required for the initial T cell priming, partly due to antigen-specific B cells being much rarer than DCs (Itano & Jenkins 2003) and, as discussed in the introduction, T cells are usually primed by DCs in the T zone before they encounter cognate B cells at the B/T border. Hong and colleagues discovered that while this model is true for immunisations with particulate antigen, immunisation with virus-like particles (VLPs) - multivalent nanoparticles, comprised of assembled viral coat proteins, leads to a potent T cell priming that requires B cells and is independent of DCs (Hong et al 2018). We used this to ask whether bypassing the need for DC-mediated priming will rescue the GC phenotype in SIGN-R1 depleted mice. We reasoned that if SIGN-R1 macrophage-depleted mice fail to mount a mature GC response due to impaired priming of Tfh cells owing to a loss of 33D1+ DC positional regulation, applying a DC-independent type of immunization should bypass this defect and rescue the GC response. We immunised CLL or PBS-L-treated mice with VLPs and analysed the GCs 11 days later. We found that in this immunisation system, the GC frequencies were similar between macrophage-sufficient and deficient animals. This result adds important support to the hypothesis that the GC collapse in the absence of SIGN-R1 cells is due to suboptimal Tfh priming by DCs.

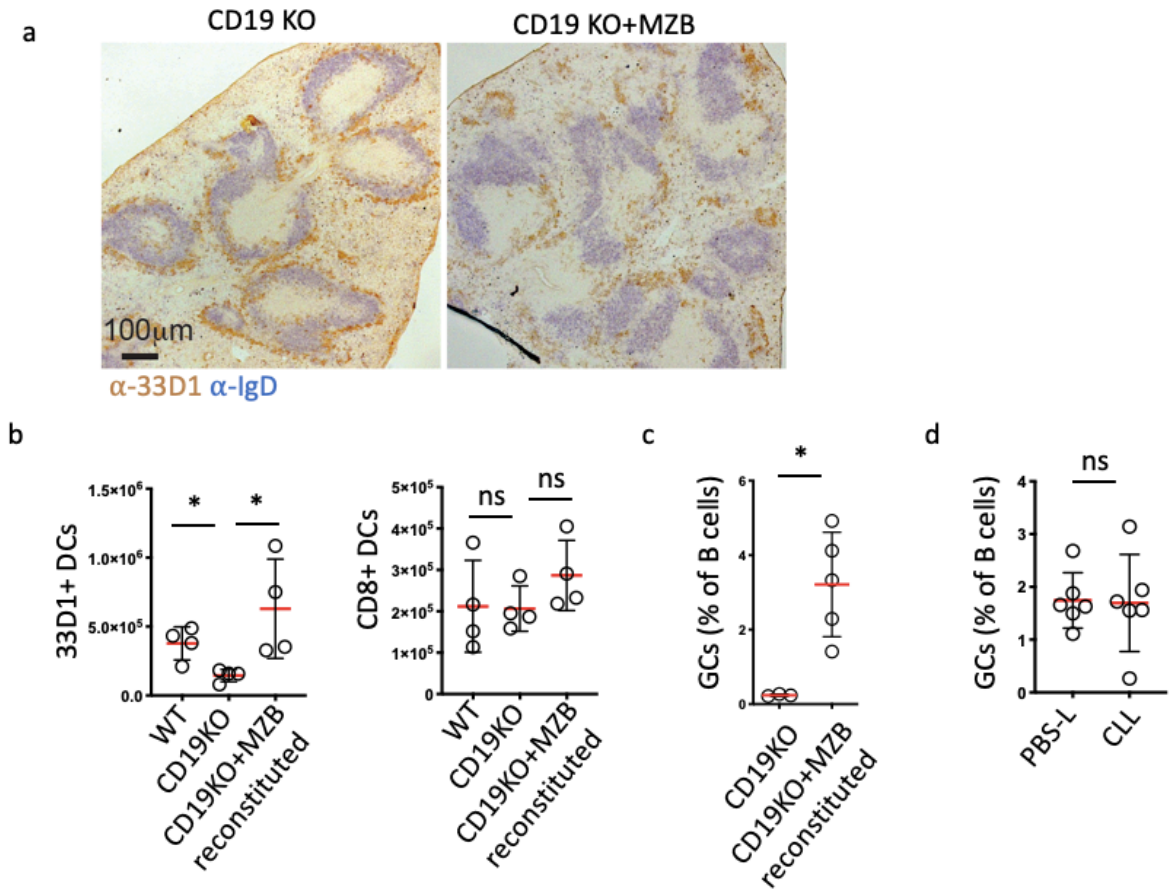


Figure 25. Aberrant DC positioning in SIGN-R1 deficient mice correlates with collapse of the GC response. *CD19KO mice were transferred or not with WT splenocytes to reconstitute the MZB cell compartment. (a) Histological analysis of frozen spleen sections stained for IgD (blue) and 33D1 (brown). Scale 100um. (b) Cell number of 33D1+ and CD8+ DC subsets. Shown one representative experiment of 3 independent repeats. (c) CD19 KO mice were reconstituted or not with WT splenocytes and 11 days later were co-transferred with MD4 and OTII cells followed by HEL-OVA immunisation the following day. Graph shows GC frequencies out of B220+ cells. Shown 1 representative experiment of 3 independent repeats, 3-5 mice per group. (d) Mice that have been treated with PBS-L or CLL 3 weeks earlier were immunized with VLPs and analysed by flow cytometry 11 days later. Graph shows frequencies of GC cells out of B220+ cells. Figure shows one out of 3 independently experiments performed.*

6.6 Conclusions and discussion

In this chapter we showed that depleting SIGN-R1 macrophages with both pharmacological and genetic approaches lead to remodelling of the localization of 33D1+ DCs, which are important for priming CD4+ T cell responses. After SIGN-R1 depletion, 33D1+ DCs were located predominantly in the splenic marginal zone instead of the interfollicular regions. While previous research has shown a dependency of 33D1+ DCs on localisation at the interfollicular regions for survival (Gatto et al 2013, Yi & Cyster 2013), when using acute depletion of the macrophages with CLL or DT we did not observe significant differences in the frequencies and numbers of 33D1+ DCs. However, the published studies used genetic models with chronic obstruction of DC localisation at these regions. Therefore, the short-term SIGN-R1 depletion in either of our systems might have been insufficient to expose a defect in DC maintenance. In agreement with this possibility, when we used CD19KO mice as a model for long-term SIGN-R1 cell depletion, the numbers and frequencies of 33D1+ DCs were significantly decreased. Notably, unlike the genetic and clodronate depletion methods, the absence of SIGN-R1 cells in CD19KO mice is specific to the spleen, where MZB cells reside. This further supports the notion that loss of local SIGN-R1 cells in the MZ compartment, rather than depletion of SIGN-R1 cells in other sites (e.g., peritoneum, LNs, etc) was a major factor that contributed to the impaired GC responses observed.

An additional important support for our hypothesis that 33D1+ DC mediated priming is defective when SIGN-R1 cells are missing, comes from our finding that upon immunising macrophage-depleted mice with VLPs, which bypasses the need for DCs in Tfh priming, we no longer observed a collapse in GCs. Taken together this data supports a previously unappreciated link between innate and adaptive immunity and

suggests that splenic SIGN-R1 macrophages regulate 33D1+ DC localisation, maintenance and function, and thus indirectly support the optimal differentiation of Tfh cells and subsequently the maintenance of the GC reaction.

While the phenotypes we describe here are reproducible among multiple SIGN-R1 depletion and immunisation systems, a mechanistic insight is needed to further understand these processes, particularly how SIGN-R1 macrophages regulate DC positioning and how DC functions are abrogated.

7 Discussion

This work provides evidence that SIGN-R1 marginal zone macrophages have a specific and non-redundant role in the regulation of germinal centre responses in the spleen. Furthermore, these findings show that SIGNR1+ MARCO+ and SIGN-R1-MARCO+ MZ macrophages, although closely related populations that occupy the same niche, can be considered distinct subsets in some respects, as they carry out specialised functions. Our data highlights that uncoupling the roles of the different MZ-resident populations as well as their developmental requirements can help us gain a better understanding of how the immune responses to blood-borne pathogens are regulated. Furthermore, we have validated the first genetic mouse model that can specifically deplete and genetically modify SIGN-R1+ cells, which could be applied to understand the organ-specific functions of SIGN-R1-expressing cells in the spleen, LNs and peritoneum.

Our data suggests that splenic SIGN-R1 macrophages regulate GC responses by maintaining optimal Tfh differentiation. Tfh maintenance is dependent on continuous

GC B cell signalling and vice versa (Baumjohann et al 2013, Gitlin et al 2014). Therefore, a defect in either population could lead to GC collapse. While our data does not definitively exclude a GC B cell-intrinsic defect in the absence of macrophages, it suggests against this possibility. This is supported by the observation that the initial activation, migration and function of pre-GCs derived from SIGN-R1 macrophage-depleted animals was intact. Furthermore, boosting Tfh help or inducing a competent Tfh cell population prior to macrophage ablation significantly improved the GC response. Thus, these results favour the possibility that SIGN-R1 macrophages regulate GC B cell differentiation indirectly by ensuring the optimal activation of Tfh responses.

Using 3 different SIGN-R1 macrophage ablation models (CLL, DT and CD19KO mice), we show that SIGN-R1 cells are essential for the ability of 33D1+ cDC2 cells to localize to the interfollicular regions in the spleen. Given the critical role of cDC2s in regulating GC B cell responses by priming Tfh cells, we hypothesise that loss of positional regulation of these cells contributes to the impaired GC B cell development observed in SIGN-R1 macrophage-depleted mice. In support of this possibility, when we used VLPs as a particulate antigen immunizing model that is known to prime Tfh cells independently of DCs, depletion of SIGN-R1 macrophages had no effect, strongly supporting the possibility that SIGN-R1 macrophages regulate this specific pathway. However, we currently do not have a clear understanding of the mechanisms involved, as our analysis shows that loss of SIGN-R1 macrophages is not associated with impaired antigen capture, upregulation of a range of activation markers or migration of 33D1+ DCs to T zones during immunization. One hypothesis is that the underlining mechanism may involve alternative activation of the DCs in the marginal zone. For example, exposure of DCs to IL-4 may enhance production of IFN-γ, which can then

promote differentiation of Th1 and Th2 cells, possibly limiting Tfh responses (Cook et al 2012). Thus, the manner by which DCs are primed can have profound effects on their subsequent capacity to drive specific T cell differentiation programs. Developing robust *in vitro* assays to assess DCs capacity to trigger Tfh cell differentiation combined with genomic approaches (e.g. single cell RNAseq) to explore whether SIGN-R1 macrophages impact the activation of 33D1+ DCs, may help to address this point.

Another interesting open question that comes up from our study is how SIGN-R1 macrophages regulate 33D1+ DCs positioning. It is possible that SIGN-R1 cells prevent 33D1+ DC migration by physically occupying the marginal zone niche and competing on local resources. Alternatively, SIGN-R1 macrophages could be actively inhibiting the migration of DCs into the marginal zone, for example by secreting repelling chemokines or by actively degrading EBI2 ligands that are potentially present within the marginal zone (Lu et al 2017). Thus, it would be interesting to test whether SIGN-R1 macrophages express the EBI2 ligand degrading enzyme, Hsd3b7 or if they secret any other relevant chemokines that may be involved. Developing better ways to analyse these cells using genomic approaches will greatly enhance the field and help to address this as well as many other important questions.

Our findings here suggest that loss of SIGN-R1 cells induces a suppression of the adaptive immune compartment. As mentioned in the introduction, the presence of SIGN-R1 in the MZ is dependent on co-localisation with MZB cells (You et al 2011). Extrinsic and innate stimuli such as LPS from blood-borne bacteria, treatment with S1PR1 antagonist, or adrenergic signalling after stroke have been shown to induce displacement of MZB cells from the marginal zone and migration into the follicles

(McCulloch et al 2017, You et al 2011). It is therefore possible that similar physiological conditions that lead to MZB displacement from the marginal zone, will also lead to a temporary 'shut down' of adaptive immunity in the spleen. A similar transient 'shut down' effect was previously observed in other components of the adaptive immune response. For example, T cell migration into the spleen is transiently obstructed following an infection or LPS administration (Chauveau et al 2020, Mueller et al 2007) and in the lymph nodes, infection-mediated depletion of subcapsular sinus macrophages prevents a response to a secondary infection (Gaya et al 2015). While the functional relevance of such a 'shut down' effect remains unknown, blocking secondary responses may enhance competition within secluded FOs to improve affinity maturation and generate higher affinity antibodies (Gaya et al 2015). This hypothesis may be particularly relevant to the spleen, where typically only a fraction of the follicles contains germinal centres, while others show no sign of activation. Thus, a transient loss of SIGN-R1 macrophages after a strong inflammatory signal could help to temporarily "shut down" follicles with ongoing responses, allowing follicles to remain "free" for activation with a new antigen.

While this work provides important insight into how the marginal zone can regulate germinal centre responses in the mouse spleen, the translatability of our results to humans is limited as there are major differences in the splenic architecture between the two species, that could result in different cell migration patterns and interactions. Similar to mice, the human spleen is subdivided into a red pulp and a white pulp compartment. However, unlike in mice, where a single T zone forms around an arterial vessel with several B cell follicles surrounding it, in the human spleen both B cell follicles and T cell zones are arranged along blood vessels like grapes on a vine, with

T zones limited to larger arteries and B cell follicles found on smaller arterioles as well. A major difference between mouse and human spleens is the border between the white and the red pulp. In humans there is no clearly defined marginal zone region as there is no marginal sinus to physically separate the follicle from the red pulp (Steiniger 2015). Instead, a "superficial zone" (sometimes referred to as outer marginal zone) surrounds B cell follicles, where a population of $IgM^{+}IgD^{+/-}CD27^{+}$ B cells resides and is believed to be the equivalent of mouse MZB cells. This MZB-like cell population however differs from mouse MZBs in that it is migratory and not unique to the spleen, found in the blood as well as other secondary lymphoid organs (Steiniger 2015), which suggests these cells do not depend on the splenic environment for maintenance (Steiniger 2015). Encompassing the superficial zone in the human spleen is the so called "perifollicular zone", that contains sheathed capillaries as well as blood-filled pockets without endothelial lining (Steiniger 2015) and also contains CD169+ macrophages, resembling mouse metallophilic macrophage localisation. MAdCAM+ stromal cells and dendritic cells are also abundant in the superficial and perifollicular zones (Steiniger et al 2001, Steiniger 2015), reminiscent of the mouse spleen, where MAdCAM+ endothelial cells line the marginal sinus with MZ-dwelling DCs in close proximity. Another major difference between the two species, that limits the direct translatability of our findings is that the human homologue of SIGN-R1, DC-SIGN, is present on human DCs and not macrophages (Figdor et al 2002, Park et al 2001). Histological analysis of human spleens suggests DC-SIGN+ DCs could potentially be found in the perifollicular region (Geijtenbeek et al 2000), resembling SIGN-R1 positioning in mice. However, more robust analysis with appropriate markers is needed to confirm their positioning relative to the follicles (Geijtenbeek et al 2000). Nevertheless, the perifollicular region of the human spleen does contain macrophages that differ phenotypically from their red pulp

counterparts (Steiniger 2015), suggesting functional specification of this population. In summary, despite the structural differences between the human and mouse spleen, there are ample similarities in terms of surface marker expression and general structural arrangement, which suggests that the findings of our research and others in mouse spleen biology can help guide the necessary future studies into human spleen as similar mechanisms and intercellular dependencies could apply in humans as well.

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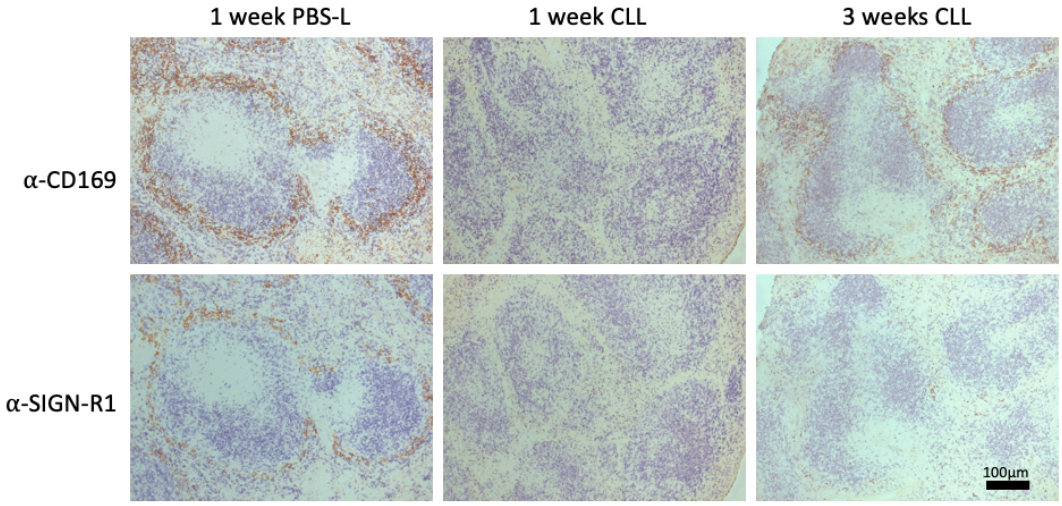
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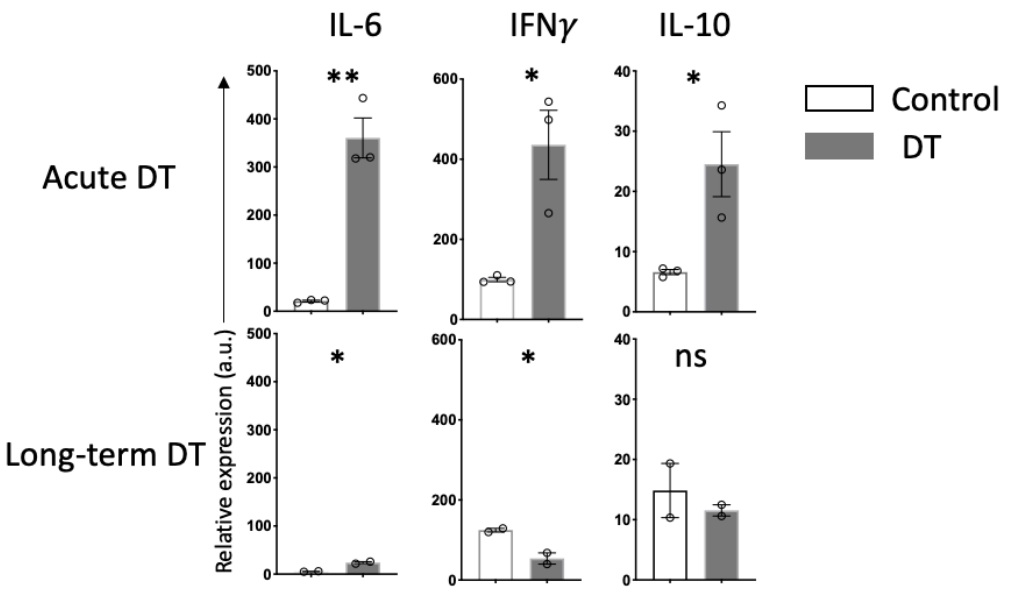
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9 Appendix

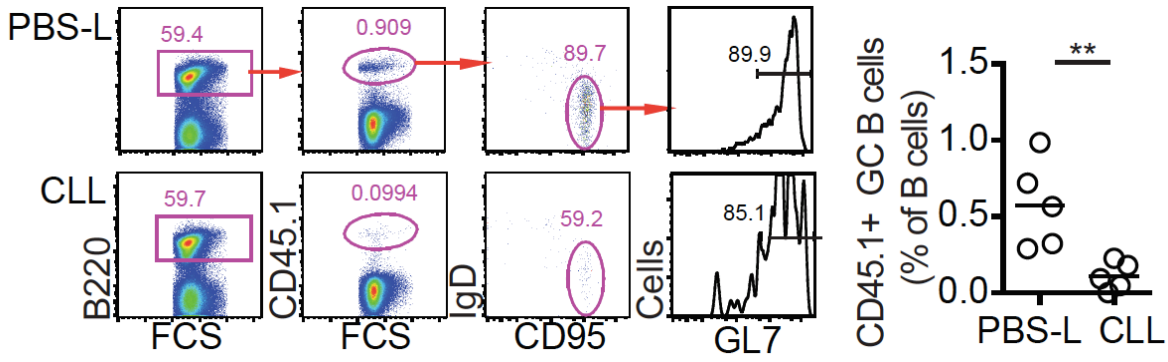


Appendix Figure 1. Macrophage repopulation kinetics after clodronate liposome-mediated depletion. *B6 mice were treated with CLL or PBS-L and 1 or 3 weeks later spleens were collected and analysed by immunohistochemistry for the expression of SIGN-R1 or CD169 (brown) and IgD (blue). Data represent one example out of 3 independent experiments performed. In each experiment, sections from at least 3 spleens per group were analysed.*



Appendix Figure 2. <u>Long-term DT treatment reduces the inflammatory response associated with acute macrophage ablation.</u>

CD169-DTR homozygous mice were treated with DT on days -3 and -1 (acute) or -6, -3 and -1 (long term) and spleens were collected on day 0. qPCR showing relative expression of IL-6, IFNγ and IL-10, normalized to GAPDH. One representative experiment of 2. The experiments were performed by Anne Chauveau.



Appendix Figure 3. Ex-vivo 'induced GC B cells' (iGCBs) were generated from MD4 B cells <u>in vitro</u>

CD45.2+ B6 mice were treated with CLL or PBS-L and 3 weeks later were immunised with SRBC. On day 4 after immunisation mice were transferred with iGCB cells (MD4-CD45.1) and boosted with SRBC-HEL one day later. Transduced B cell incorporation was assessed on day 7 after the boost. Percentage of CD45.1+ GC B cells within the follicular compartment recovered from recipients. Experiment represents 1 out of 2 experiments, 4-5 mice in each group. Experiments performed by Andrew MacLean.